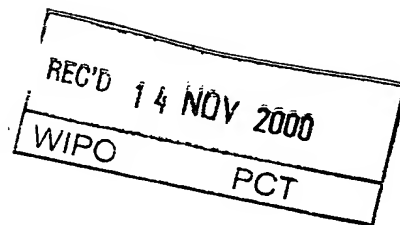


Office de la propriété
intellectuelle
du Canada

Un organisme
d'Industrie Canada

Canadian
Intellectual Property
Office

An Agency of
Industry Canada



*Bureau canadien
des brevets*
Certification

La présente atteste que les documents
ci-joints, dont la liste figure ci-dessous,
sont des copies authentiques des docu-
ments déposés au Bureau des brevets.

*Canadian Patent
Office*
Certification

This is to certify that the documents
attached hereto and identified below are
true copies of the documents on file in
the Patent Office.

Specification and Drawings, as originally filed, with Application for Patent Serial No:
2,283,538, on September 30, 1999, by STANLEY NG, MUN HON, IM AND JI-
ZHONG ZHANG, for "New Hev Antigenic Peptide and Methods".

**PRIORITY
DOCUMENT**
SUBMITTED OR TRANSMITTED IN
COMPLIANCE WITH RULE 17.1(a) OR (b)

S. H. Gregoire
Agent certificateur/Certifying Officer

September 22, 2000

Date

Canada

(CIPO 68)

OPIC



CIPO

ABSTRACT

A highly immunoreactive viral peptide, E2, of the hepatitis E virus (HEV) genome, derived from the carboxyl terminal end region of ORF2, is disclosed. The ORF2 peptide, expressed either as a glutathione S-transferase (GST) fusion peptide (GE2) or as the viral peptide (E2) cleaved from sepharose-bound GE2, proved to be highly reactive with sera from patients having current or past infection with HEV. A special feature of E2 is that it is a conformational antigenic determinant generated by intramolecular interactions between monomers of the peptide to form homodimers. The immunoreactivity is strictly conformational in nature and is only functional when the E2 peptide is in a dimeric form such that immunoreactivity is lost upon dissociation of the dimer. Moreover, diagnostic methods useful in detecting HEV infection and an antigen vaccine composition effective in preventing hepatitis E virus infection in which E2 is utilized are also disclosed.

NEW HEV ANTIGENIC PEPTIDE AND METHODS

FIELD OF THE INVENTION

5 The present invention relates to an antigenic peptide, E2, cloned from the genome of a Chinese strain of hepatitis E virus (HEV) and its subsequent utilization in the development of reliable diagnostic methods for the detection of HEV and vaccine compositions for the prevention of HEV in humans.

10

REFERENCES

Aye, T.T., T. Uchida, X.Z. Ma, F. Lida, T. Shikata, H. Zhuang and M. Wink. 1992. *Complete Nucleotide Sequence of a Hepatitis E Virus Isolated from the Xinjiang Epidemic.* Nucleic Acids Res., 20:3512.

15

Aye, T.T., T. Uchida, X.Z. Ma, F. Lida, T. Shikata, M. Ichikawa, T. Rikihisa, and M. Wink. 1993. *Sequence and Gene Structure of the Hepatitis Virus Isolated from Myanmar.* Virus Genes. 7:95-109.

20

Balayan, M.S., A.G. Andjaparidze, S.S. Savinskaya, E.S. Ketiladze, D.M. Braginski, A.P. Savaionon, and V.F. Polescschuk. 1983. *Evidence for a Virus in Non-A, Non-B Hepatitis Transmitted Via the Faecal Oral Route.* Intervirology. 20:23.

25

Belabbes, E.H., A. Bourgurmouh, A. Benatallah, and G. Illoul. 1985. *Epidemic Non-A, Non-B Viral Hepatitis in Algeria: Strong Evidence for is Spending by Water.* J. Med. Virol. 16:257-263.

30

Beril, C., J.M. Crance, F. Leguyader, V. Apaire-Marchais, F. Leveque, M. Albert, M.A. Goraguer, L. Schwartzbrod, and S. Billaudel. 1996. *Study of Viral and Bacterial Indicators in Cockle and Mussels.* Marine Pollution Bulletin. 32:404-409.

Berke, T., B. Golding, X. Jiang, W.C. David, M. Wolfaardt, A.W. Smith, and D.O. Matson. 1997. *Phylogenetic Analysis of the Caliciviruses.* J. Med. Virol. 52:419-424.

Bradley, D.W. and M.A. Purdy. 1994. *Molecular and Serological Characteristics of Hepatitis E Virus*. Pages 42-45. In Nishioka K., H. Suzuki, S. Mishiro, and T. Od (Ed.), *Viral Hepatitis and Liver Disease*, Toyko.

- 5 Bradley, D.W. and M.S. Balayan. 1988. *Virus of Enterically Transmitted Non-A, Non-B Hepatitis*. *Lancet*. 1:819.

- 10 Coursaget, P., Y. Buisson, N. Depril, P.L. Canne, M. Chavaud, C. Molinie, and R. Roue. 1993. *Mapping of Linear B Cell Epitopes on Open Reading Frames 2 and 3 Encoded Proteins of Hepatitis E Virus Using Synthetic Peptides*. *FEMS Microbiol. Lett.* 109:251-256.

Demeke, T. and R.P. Adams. 1992. *The Effects of Plant Polysaccharides and Buffer Additives on PCR Biotechniques*. 12:332-334.

- 15 Dilawari, J.B., K. Singh, Y.K. Chawla, G.N. Ramesh, A. Chauhan, S.R. Bhusnurmath, T.R. Sharma, and C.S. Sokhey. 1994. *Hepatitis E Virus: Epidemiological, Clinical and Serological Studies of North Indian Epidemic*. *Indian J. Gastroenterol.* 13:44-48.

- 20 Hau, C.H., T.T. Hien, N.T. Tien, H.B. Khiem, P.K. Sac, V.T. Nhung, R.P. Larasati, K. Laras, M.P. Putri, R. Doss, K.C. Hyams, and A.L. Corwin. 1999. *Prevalence of Enteric Hepatitis A and E Viruses in the Mekong River Delta Region of Vietnam*. *Am. J. Trop. Med. Hyg.* 60:277-280.

- 25 Huang, C.C., D. Nguyen, J. Fernandez, K. Yun, K.E. Fry, D.W. Bradley, A.W. Tam, and G.R. Reyes. 1992. *Molecular Cloning and Sequencing of the Mexican Isolate of Hepatitis E Virus (HEV)*. *Virology*. 191:550-558.

- 30 Hussaini, S.H., S.J. Skidmore, P. Richardson, L.M. Sherratt, B.T. Cooper, J.G. O'Grady. 1997. *Severe Hepatitis E Infection During Pregnancy*. *J. Viral. Hepat.* 4:51-54.

Imai, H., O. Yamada, S. Morita, S. Suehiro, and T. Kurimura. 1992. *Detection of HIV-1 RNA in Heparinized Plasma of HIV-1 Seropositive Individuals*. *J. Virol. Methods*. 36:181-184.

- Khudyakov, Y.E., M.O. Favorov, D.L. Jue, T.K. Hine, and H.A. Field. 1994. *Immunodominant Antigenic Regions in a Structural Protein of the Hepatitis E Virus*. *Virology*. 198:390-393.
- 5 Khudyakov., Y.E., N.S. Khudyakov, H.A. Field, D. Jue, C. Starling, M.O. Favorov, K. Krawczynski, L. Polish, E. Mast, and H. Margolis. 1993. *Epitope Mapping in Protein of Hepatitis E Virus*. *Virology*. 194:89-96.
- 10 Kolk, A.H., A.R. Schuitema, S. Kuijper, J. van Leeuwen, P.W. Hermans, J.D. van Embden, and R.A. Hartskeerl. 1992. *Detection of Mycobacterium Tuberculosis in Clinical Samples by Using Polymerase Chain Reaction and a Nonradioactive Detection System*. *J. Clin. Microbiol.* 30:2567-2575.
- 15 Myint, H., M.M. Soe, T. Khin, T.M. Myint, and T.M. Tin. 1985. *A Clinical and Epidemiological Study of an Epidemic of Non-A, Non-B Hepatitis in Rangoon*. *Am. J. Trop. Med. Hyg.* 34:1183-1189.
- 20 Panda, S.K., R. Datta, J. Kaur, A.J. Zuckerman, and N.C. Nayak. 1989. *Enterically Transmitted Non-A, Non-B Hepatitis: Recovery of Virus-Like Particle From an Epidemic in South Delhi and Transmission of Studies in Rhesus Monkey*. *Hepatology*. 104:66-72.
- Reyes, G.R., C.C. Huang, A.W. Tam, and M.A. Purdy. 1993. *Molecular Organization and Replication of Hepatitis E Virus (HEV)*. *Arch. Virol. Suppl.* 7:15-25.
- 25 Sergeev, N.W., E.A. Paktoris, W.A. Anaev, G.A. Sinjko, A.I. Antinova, and E.P. Semenov. 1957. *General Characteristics of Botkin's Disease Occuring in Kirgiz Republic of USSR in 1955-1956*. *Soviet Health Care Kirgizii*. 5:13-16.
- 30 Smith, D.B., and K.S. Johnson. 1988. *Single-step Purification of Polypeptides Expressed in Escherichia Coli as Fusions With Glutathione S-Transferase*. *Gene*. 67:31-40.
- Tam, A.W., M.M. Smith, M.E. Guerra, C.C. Huang, D.W. Bradley, K.E. Fry, and G. R. Reyes. 1991. *Hepatitis E Virus (HEV) Molecular Cloning and Sequencing of Full-Length Viral Genome*. *Virology*. 185:120-131.

- Tsai, Y.L., C.J. Palmer, and L.R. Sangermano. 1993. *Detection of Escherichia Coli in Sewage and Sludge by Polymerase Chain Reaction*. Appl. Environ. Microbiol. 59:353-357.
- 5 Tsai, Y.L., and B.H. Olson. 1992. *Detection of Low Numbers of Bacterial Cells in Soils and Sediments by Polymerase Chain Reaction*. Appl. Environ. Microbiol. 58:754-757.
- Tsai, Y.L. and B.H. Olson. 1992. *Rapid Method for Separation of Bacterial DNA From Humic Substances in Sediments for Polymerase Chain Reaction*. Appl. Environ. Microbiol.
10 58:2292-2295.
- Tsarev, S.A., T.S. Tsareva, S.U. Emerson, S. Govindarajan, M. Shapiro, J.L. Gerin, and R.H. Purcell. 1994. *Successful Passive and Active Immunization of Cynomolgus Monkeys Against Hepatitis E*. Proc. Natl. Acad. Sci. U.S.A. 91:10198-10202.
- 15 Tsarev, S.A., T.S. Tsareva, S.U. Emerson, S. Govindarajan, M. Shapiro, J.L. Gerin, and R.H. Purcell. 1997. *Recombinant Vaccine Against Hepatitis E: Dose Response and Protection Against Heterologous Challenge*. Vaccine. 15:1834-1838.
- 20 Tsega, E., B-G. Hanson, K. Krawczynky, and E. Nordenfelt. 1992. *Acute Sporadic Viral Hepatitis in Ethiopia: Causes, Risk Factors and Effects of Pregnancy*. Clin. Infec. Dis. 14:961-965.
- Visvanathan, R. 1957. *Infectious Hepatitis in Delhi (1955-1956): A Critical Study: Epidemiology*. Indian J. Med. Res. (Suppl.). 45:1-30.
- 25 Wong, D.C., R.H. Purcell, M.A. Sreenivasan, S.R. Prasad, and K.M. Pavri. 1980. *Epidemic and Endemic Hepatitis in India: Evidence for Non-A, Non-B Hepatitis Virus Etiology*. Lancet. 2:882-885.
- 30 Zhuang, H., F. Li, Q.Y. Wang, X.X. Guo, T.T. Ai, X.Z. Ma, and H.J. Dong. 1992. *Detection of HEV RNA in Bile of Macaca Multta by Polymerase Chain Reaction*. J. Chinese Exp. Clin. Virol. 6(2):111-114.

BACKGROUND OF THE INVENTION

Hepatitis E virus (HEV) was first discovered in 1983 as a cause of enterically transmitted hepatitis (Balayan et al., 1983). The full length viral genome was first cloned and sequenced in 1991, and it was found to be a single-stranded positive sense unenveloped RNA (Tam et al., 1991). Although morphologically resembling members of the Caliciviridae (Bradley et al., 1988; Huang et al., 1992; Panda et al., 1989), it has a distinct genomic organization (Berke et al., 1997). Based on sequence analysis, the 7.2 kb viral genome is predicted to contain three open reading frames (ORF) (Figure 1) (Tam et al., 1991; Aye et al., 1992; Aye et al., 1993; Huang et al., 1992; Reyes et al., 1993). Non-structural viral proteins are encoded as a polyprotein by ORF1 located at the 5' terminus of the viral genome. ORF2 is located at the 3' end of the genome and encodes a viral capsid protein. The 5' end of ORF3 has one base overlapping with the 3' end of ORF1 and the 3' end has 339 bases overlapping with the 5' end of ORF2. ORF3 is believed to code for another structural protein whose function is still unknown. Linear antigenic epitopes have been located in ORF2 and ORF3 by epitope mapping and study of recombinant peptides (Coursaget et al., 1993; Khudyakov et al., 1993; Khudyakov et al., 1994), however, no conformational antigenic determinants of HEV have been described to date.

Hepatitis E principally occurs in developing countries in both epidemic and sporadic forms. Several large outbreaks of hepatitis E occurred in the 1950's to 1980's caused by sewage-polluted drinking water (Visvanathan, 1957; Wong et al., 1980; Myint et al., 1985; Belabbes et al., 1985; Hau et al., 1999). The infection is usually self-limiting, but there are reports of serious complications when infection occurs during pregnancy (Tsega et al., 1992; Dilawari et al., 1994; Hussaini et al., 1997). As prevention is an important aspect of combatting infection, reliable detection of the virus in environmental specimens is an essential requirement for public health and environmental protection. Traditional methods available for collecting and concentrating virus particles have several known disadvantages that limit the investigation of the vectors and reservoirs of HEV. Two of the most common methods are adsorption and centrifugation.

In the adsorption method, viruses are first concentrated by adsorption to microporous

filters and subsequently eluted with large volumes of eluent. However, this technique also effectively concentrates a variety of other solutes, such as humic acids and proteins, which may interfere with the detection of viruses. In particular, many naturally occurring inorganic and organic solutes inhibit the nucleic acid polymerases used for amplification of target genomes (reverse transcriptase and Taq polymerase) (Tsai et al., 1992a; 1992b; 1993). Nucleases and proteases may also degrade virus genomes before they can be amplified. In addition, various proteins, carbohydrates, and other organic compounds may bind-magnesium-ions-and-nucleotides-required-by-nucleic-acid polymerases and some solutes may be toxic to these polymerases (Demeke and Adams, 1992; Imai et al., 1992; Kolk et al., 1992).

In the centrifugation method, the sample is homogenized and then centrifuged repeatedly. During the process, polyethylene glycol (PEG) is added to the supernatant and then centrifuged again. The final pellet is resuspended in buffer, however the final concentrate still contains toxic substances which may interfere with subsequent methods such as cell culturing, reverse transcription and polymerase chain reaction (PCR) (Beril et al., 1996). As a result, the concentrate needs to undergo detoxification by gel filtration on sephadex. As a result of the disadvantages described above, a more sensitive and reliable technique needs to be established for the purpose of detecting HEV in environmental specimens.

Moreover, it has hitherto not been possible to develop a vaccine against HEV infection using live attenuated or killed viral particles because of the difficulty in propagating the virus in cultured cells. HEV peptides, especially those specified by the structural genes of the virus, have found useful applications as diagnostic reagents and some of them are able to afford protection against the virus (Tsarev et al., 1994; Tsarev et al., 1997).

Accordingly, the present invention provides a highly immunoreactive viral peptide, E2, of the hepatitis E virus (HEV) genome which is derived from the carboxyl terminal end region of ORF2 and proven to be highly reactive with sera from patients having current or past infection with HEV. Accordingly, diagnostic methods useful in detecting HEV infection and an antigen vaccine composition effective in preventing hepatitis E virus infection in which E2 is utilized are also provided.

SUMMARY OF THE INVENTION

An object of the invention is to provide a HEV peptide, E2, which undergoes conformational changes brought about by interactions between E2 monomers to form antigenic determinants which are highly immunoreactive with sera from individuals infected with the hepatitis E virus.

~~Another object of the invention to provide an improved and more reliable diagnostic method utilizing the E2 peptide, and specific polyclonal antibodies directed against E2, in an immune capture (IC) technique, which allows HEV particles to be captured and concentrated through specific Ag-Ab affinity interactions and which overcomes particular disadvantages prevalent in the use of known methods.~~

Still another object of the invention is to provide an ELISA method utilizing the E2 antigen for determining current and past infection of HEV through the detection of IgM and IgG antibodies, respectively, in clinical and biological specimens.

~~Yet another object of the invention is to provide a vaccine composition comprising the E2 peptide which is effective in preventing HEV following immunization.~~

According to the invention there is provided a highly immunoreactive viral peptide, E2, of the hepatitis E virus (HEV) genome which is derived from the carboxyl terminal end region of ORF2 and contains an amino acid sequence identified as SEQ I.D. NO: 3.

Another aspect of the invention provides a diagnostic method and kit useful in detecting HEV infection in test individuals in which antiserum raised against the E2 peptide is coated on a solid support and then examined for the presence of bound antibody following contact with a test sample.

Still another aspect of the invention provides a vaccine composition effective in preventing hepatitis E virus infection which comprises the viral peptide, E2, and a pharmacologically acceptable carrier.

More specifically, various embodiments of the invention include the use of an E2 peptide containing an amino acid sequence represented by SEQ ID NO: 3, homologous

s qu nces th rewith, and fragments, analogs, polymers and chimeras th reof. In the pr sent invention, th expression, purification and characterization of the highly immunoreactive structural peptide from HEV is described in which a new conformational antigenic determinant is generated by intramolecular interactions between HEV capsid peptides. Translated from the carboxyl domain of the ORF2 sequence, the E2 protein was predicted to be 267 aa peptide corresponding to the carboxyl domain of the full length protein. Based on similar peptide mapping studies (Khudyakov et al., 1993; Khudyakov et al., 1994), it was also expected that the peptide would contain linear epitopes at its N and C terminus. However, a frame-shift mutation in the cloned sequence caused translation to terminate prematurely at a new termination codon upstream giving rise instead to a smaller peptide of 215 aa. Its antigenic activity is highly recognized by HEV reactive human sera and has been largely attributed to antigenic determinants generated as a result of intramolecular interactions and conformational changes between monomers to form homodimers.

The same or similar conformational antigenic determinants also appear to be represented on the viral capsid, such that hyperimmune sera specifically reactive against the E2 protein can effect immune capture of the virus particles. These results suggest that interaction between the HEV ORF2 capsid proteins form an unrecognized, conformational antigenic determinant of potential importance in the development of diagnostic tests and vaccines for HEV infection. Moreover, this antigenic domain is distinct from previously identified linear epitodes within the N terminal domain because a similar conformational antigenic determinant has not been previously described or predicted by peptide mapping.

BRIEF DESCRIPTION OF THE DRAWINGS

In the following description, the invention will be explained in detail with the aid of the accompanying figures which illustrate preferred embodiments of the present invention and in which:

Figure 1 shows the genomic map of HEV with the particular arrangement of the open reading frames ORF1, ORF2 and ORF3 and the approximate coding region for peptide, E2 and E3;

Figures 2A to 2D provide the nucleotide sequences for ORF2 of Chinese HEV strain and the e2 fragment derived therefrom; the single base pair deletion being indicated by a box;

Figure 3 provides the nucleotide sequence for ORF3 of Chinese HEV strain and the e3
5 fragment derived therefrom;

Figure 4 provides the amino acid sequence of the E2 peptide encoded by e2;

Figure 5 provides the amino acid sequence of the E3 protein encoded by e3;

10

Figure 6 shows the characterization of recombinant plasmids carrying the HEV genomic sequences. A 821 bp insert containing a 810 bp sequence of ORF2 (Lane 2) and a 124 bp insert containing a 114 bp sequence of ORF3 (Lane 3) of the HEV genome were obtained by digestion of recombinant pGEX₂₀ plasmids with BamHI and EcoRI. The
15 molecular weight of these products were compared with markers (Lane 1);

20

Figure 7 shows the characterization of purified HEV peptides expressed from ORF2. Purified HEV GST fusion peptide, GE2 (Lanes 1 and 2), the viral peptide E2 (Lanes 3 and 4) and GST (Lane 5) were subjected to analysis by (A) PAGE and (B) Western blotting using a GST-specific antiserum and (C) a pooled human HEV reactive human serum. The
20 molecular weight of these products were compared with markers (Lane 1). The GE2 and E2 peptides (Lanes 1 and 3) were heated at 100°C for 3 minutes (Lanes 2 and 4) prior to analysis. The molecular weight of these products were compared with markers (Lane 6);

25

Figure 8 shows the characterization of the GST fusion peptide, GE3, expressed from ORF3 of the HEV genome. The GE3 peptide (Lane 1), which was heated at 100°C for 3 minutes (Lane 2), and GST (Lane 3) were subjected to analysis by (A) PAGE and (B) Western blotting using a GST specific antiserum and (C) a pooled human serum. The
30 molecular weight of these products were compared with markers (Lane 4);

35

Figure 9 shows the treatment of the HEV peptide, E2, with 8 M urea. Purified E2 was treated with 8 M urea for 1 hour at 4°C (Lane 1), 20°C (Lane 2), 37°C (Lane 3) and 45°C (Lane 4). Purified E2 was also treated with 8 M urea at 45°C for 1 hour followed by dialysis against 1xPBS overnight (Lane 5). These samples were subjected to (A) PAGE

and (B) Western blotting using a pooled human serum;

Figure 10 illustrates the production of EIA with HEV peptides. Microplates were coated with predetermined optimum concentrations of a purified preparation of E2 or E3. These peptide preparations were analyzed by PAGE (left lane) and Western blotting using human sera which are reactive (solid circle), weakly reactive (hatched circle), or not reactive (open circle) against the corresponding viral peptides. The same sera was titrated in microplates coated with the respective peptides;

Figure 11 illustrates the determination of HEV antibodies by EIA and Western blotting. Sera from 96 hepatitis patients were tested at 1:100 dilution by EIA produced with purified (A) E2 or (B) E3. The patients' sera was previously tested by Western blotting against the same preparations of viral peptides and found to be reactive (solid bar), weakly reactive (hatched bar) or not reactive (open bar) against the respective peptides;

Figure 12 illustrates the time distribution of HEV antibodies after the onset of hepatitis. E2 specific (A) IgG and (B) IgM and E3 specific (C) IgG and (D) IgM antibodies in sera from 96 hepatitis patients were determined by EIA produced with the respective HEV peptides. The results were compared with the lengths of time after disease onset when these sera were taken. Cut-off OD values (dotted line) were 0.37 for E2 IgG, 0.40 for E2 IgM, 0.73 for E3 IgG and 0.53 for E3 IgM;

Figure 13 illustrates the determination of antibodies by three EIAs of distinct HEV specificity. HEV specific antibodies in sera from 86 hepatitis patients were determined by a commercially available assay (GeneLab, Singapore). The results were compared with those obtained, as in Figure 10, by assay specific for E3 (A, B and C) or specific for E2 (D, E and F). Previously performed Western blotting showed that 32 of these sera were reactive against E2 and E3 (A and D), 14 were reactive against E2 alone (B and E) and 40 were not reactive against either of these peptides (C and F). Cut-off OD values (dotted lines) were 0.6 for the commercial assay, 0.37 for E2 specific IgG and 0.73 for E3 specific IgG;

Figure 14 illustrates the specificity of HEV primers in RT-PCR using a specimen with HEV (Lane 2), HAV (Lane 3), caliciviruses (Lanes 4 and 5) and enteroviruses (Lanes 6 and 7), the molecular weight of these products being compared with markers (Lane 1);

Figure 15 illustrates immune capture of HEV. Polystyrene paddles separately coated with (A) an E2-specific antiserum or (B) pre-immune serum from the same animal were reacted with 4.5 ml samples containing a serially-diluted bile containing HEV. After washing, HEV bound to the paddles was detected by RT-PCR. The molecular weight of these products were compared with markers (left lane);

Figure 16 illustrates the purity and antigenic activity of a preparation of HEV peptide used for immunization. Purified E2 used for immunization was heated for 10 minutes at 100°C. The denatured peptide was mixed with unheated native peptide in equal proportions. The antigenicity of the preparation was analysed by (A) PAGE and the antigenicity by (B) immune blotting using an HEV reactive human serum (Lane 1) and a non-reactive human serum (Lane 2);

Figure 17 illustrates the immunization of Macaque monkeys with recombinant HEV peptides. Three adult monkeys (M1, M2, M3) were injected with 4 weekly intra-muscular doses each containing 100 µg of purified E2. Sera was obtained from the animal before immunization (Lane 5) and 2 weeks after immunization. The post-immunization sera was titrated by immune blotting against the 23 kD heat denatured E2 monomer and the 42 kD native E2 dimer (Figure 17A, Lanes 1 to 4). The post-immunization serum from M1 was titrated at serum dilutions of 1:4,000 to 1:64,000, M2 at 1:100 to 1:6,400 and M3 at 1:250 to 1:16,000. The serum samples were further tested at 1:100 dilution by immune blotting against the 30 kD GST fusion protein of another HEV peptide, E3 (Figure 17B) and by EIA using a commercial assay (Figure 17C);

Figure 18 illustrates the genomic dose of challenging virus. Animals were inoculated with 1 ml of a 1:100 dilution of a stock HEV. A genomic dose of the virus inoculated was determined by RT-PCR in serially diluted aliquots of the virus preparation as described in the section entitled "Materials and Methods";

Figure 19 illustrates the antibody response to the HEV challenge. Plasma samples obtained from the immunized and non-immunized control animals before and at the indicated times after the HEV challenge were tested by immune blotting against purified E2 and E3 and by EIA using a commercial assay;

Figure 20 illustrates an anti-E2 IgG response in Rhesus Macaque for (A) animals

immunized with the E2 peptide and (B) non-immunized controls; and

Figure 21 illustrates the detection of HEV antigen in monkey stool utilizing the sandwich ELISA for (A) the immunization group, and (B) the control group.

5

DETAILED DESCRIPTION OF THE INVENTION

(A) Definitions

10

The terms defined below have the following meaning:

2. **e2** is the cloned cDNA fragment which corresponds to position 6326 to 7136 of the genome of the Chinese HEV strain (DDBJ Accession No. D11092).

15

3. **E2** is the peptide encoded by e2.

4. **e3** is the cloned cDNA fragment which corresponds to position 5364 to 5477 of the genome of the Chinese HEV strain (DDBJ Accession No. D11092).

20

5. **E3** is the peptide encoded by e3.

6. **Glutathione S-Transferase (GST) Fusion Protein (GST)** is produced from a recombinant molecule in which a selected gene is linked to the 3' end of the GST gene.

25

7. **Hepatitis E Virus (HEV)** is a single stranded positive RNA virus morphologically similar to members of the calcivirus. It can cause sporadic cases or endemic outbreaks of hepatitis, and is serologically distinct from hepatitis A virus (HAV), hepatitis B virus (HBV), hepatitis C virus (HCV) and hepatitis D virus (HDV).

30

8. **Homodimer** is a molecule formed by the union of two identical monomers.

(B) HEV Genomic Sequences

The HEV genomic sequences shown in Figures 2A to 2D and Figure 3 correspond to the ORF2 and ORF3 regions, respectively. Similarly, the peptide sequences shown in Figures 4 and 5 correspond to the ORF2 and ORF3 regions, respectively. Accordingly, the genomic sequences listings shown are as follows:

SEQ ID No. 1 is the sequence of the cloned DNA fragment e2, which corresponds to position 6326 to 7136 of the genome of the Chinese HEV strain (DDBJ Accession No. D11092).

SEQ ID No. 2 is the sequence of the cloned DNA fragment e3, which corresponds to position 5384 to 5477 of the genome of the Chinese HEV strain (DDBJ Accession No. D11092).

SEQ ID No. 3 is the amino acid sequence of peptide E2.

SEQ ID No. 4 is the amino acid sequence of peptide E3.

(C) HEV Peptide Antigens E2 and E3

1. Expression of the HEV Structural Gene as GST Fusion Peptides

A 114 bp region from the 3' end of the ORF3 sequence and a 811 bp region from the 3' end of the ORF2 sequence located within the genome of a Chinese strain D11092 of HEV were cloned and amplified by reverse transcription-PCR. These sequences were subsequently ligated to the BamHI and EcoRI cloning sites on the pGEX vector. The cloned viral genes were recovered by digestion of the respective plasmids with EcoRI and BamHI (Figure 6, Lanes 2 and 3) and subjected to sequence analysis. The analysis located the 114 bp to position 5364 to 5477 on the viral genome. It was predicted to specify a 37 aa peptide, E3, with a MW of 3.9 kD. The 811 bp sequence was located at position 6326 to 7136 and the analysis revealed a single base pair deletion at position 6957, presumably due to a PCR amplification error. The resulting frameshift was predicted to cause the translation to terminate prematurely at a new stop codon at position

6968, giving a smaller than expected peptide of 213 aa, E2, with a MW of 23 kD, instead of 267 aa as initially expected.

The nucleotide sequences shown in Figures 2A to 2D and 3 correspond to the ORF-2 and ORF-3 regions, respectively, of the Chinese strain of HEV. The region corresponding to ORF2 has SEQ ID No. 1 and the region corresponding to ORF3 has SEQ ID No. 2.

The amino acid sequences shown in Figures 4 and 5 correspond to the ORF-2 and ORF-3 regions, respectively, of the Chinese strain of HEV. The region corresponding to ORF2 has SEQ ID No. 3 and the region corresponding to ORF3 has SEQ ID No. 4.

2. Characterization of the HEV ORF2 and ORF3 Specified Proteins

The sequences cloned from both the ORF-2 and ORF-3 regions of the HEV genome were expressed as Glutathione S-transferase (GST) fusion peptides, GE2 and GE3, respectively. This expression system gave a high yield of the viral peptides and permitted efficient purification when using the Glutathione Sepharose-4B system. This method yielded about 2 mg of purified GE2 and 7 mg of GE3 from one litre of bacterial culture. Alternatively, GE2 bound to sepharose-4B could be eluted using thrombin, which cleaved the fusion protein at the C terminus of GST to release the viral peptide E2. This procedure yielded about 1 mg of the purified E2 peptide from one litre of bacterial culture.

The purified preparations of GE2 and E2 were characterized by PAGE and Western blotting (Figure 7). Purified GE2 was resolved as a major band in a dimeric form with a MW of 92 kD (Figure 7A, Lane 1) which became dissociated into a 49 kD band after the sample had been heated at 100°C for 3 minutes (Figure 7A, Lane 2). Both the dimeric and monomeric forms of GE2 were recognized by anti-GST serum in the corresponding Western blot (Figure 7B, Lanes 1 and 2). It was noted that the 49 kD GE2 monomer, one of the minor components in this preparation, was only reactive with anti-GST, but not with the human serum (Figure 7C, Lane 1). When GE2 was heated at 100°C for 3 minutes, the 49 kD monomer became the major band, but its antigenicity against human HEV sera was markedly reduced (Figure 7C, Lane 2). The E2 peptide, which was purified by digestion of the bound fusion peptide with thrombin, also naturally formed a dimer of 46 kD which became dissociated into a 23 kD monomer after heating at 100°C for 3 minutes (Figure 7A, Lanes 3 and 4). Neither the dimeric nor the monomeric forms of E2

were recognized by anti-GST serum (Figure 7B). Furthermore, only the dimeric form of E2 was reactive with the human serum (Figure 7C).

The purified GST fusion protein expressed from ORF3 (GE3) was also characterized by PAGE and Western blotting (Figure 8). The GE3 peptide occurred as a monomer which migrated as a band with the expected MW of 30 kD. Western blotting showed that the GST fusion protein was reactive with both the anti-GST antiserum (Figure 8B) and the pooled HEV human serum (Figure 8C). However, in contrast to E2, the reactivity of GE3 was associated only with a monomeric form since its antigenic activity was not affected by heating. Therefore, it is likely that such activity is attributed, at least partly, to epitopes located by previous peptide mapping studies to the carboxyl terminus of the ORF3 specified full length protein.

3. Dimerization of the HEV E2 Peptide

An outstanding feature of E2 is that it forms homodimers under physiological conditions and that the antigenic activity of this peptide is attributed to antigenic determinants presumably arising from conformational changes brought about by the dimeric form. Furthermore, the antigenic activity is abrogated upon dissociation of the dimer but the activity can be restored upon reconstitution of the dimer from the monomeric form.

When purified E2 was subjected to treatment with 8 M urea at different temperatures for 1 hour, an increased dissociation of the E2 dimer into its monomeric form was effected by the treatment at a temperature of 37°C (Figure 9A, Lane 3) and complete dissociation was effected at 45°C (Figure 9A, Lane 4). In the corresponding Western blot (Figure 9B) using HEV reactive human serum, it was shown that dissociation of the dimeric form was associated with a loss of antigenic activity (Figure 9B, Lane 4). Recovery of antigenic activity was achieved when the monomer, obtained by urea treatment at 45°C, had reassociated to the dimeric form following overnight dialysis against PBS (Figure 9B, Lane 5).

These results suggest that E2 monomers naturally interact with one another to form dimers and that the antigenic determinants recognized by HEV reactive human serum might arise as a result of such interactions since antibodies against the monomeric form of E2 could not be detected. Accordingly, the immunoreactive region appears to be

conformational in nature and is only functional when the E2 peptide is in a dimeric form. Dissociation of the dimeric form of the peptide was associated with a loss of antigenic activity which could be partially restored by reconstitution of the dimer upon dialysis of the urea-treated samples.

5

4. Antigenic Specificity of HEV Structural Peptides

Western blotting was used to evaluate the antigenic specificity of the E2 and GE3 peptides using sera from 21 healthy donors, 13 confirmed HEV-infected patients and 96 sera from patients with non-A, B and C hepatitis (Table 1). Overall, 75 sera were reactive, one or both peptides being either E2 or both E2 and GE3, while only one of the sera was exclusively reactive with GE3. Of the positive sera, 47 were reactive to both the GE3 and E2 peptides and 27 were reactive with E2 alone in its dimeric form, not its monomeric form. In particular, with hepatitis E patients, 12 out of 13 sera were reactive with E2 and/or GE3 while only 3 of 21 sera from healthy blood donors were similarly reactive (Chi square test was $p < 0.00004$).

15

Table 1
Detection of HEV Antibodies in Human Sera by Western Blotting

20

Antibody (IgG) Profiles		Sera Number Corresponding to Antibody Profiles		
E2	GE3	Hepatitis E Patients	Non-A, B and C Hepatitis Patients	Healthy Blood Donors
+	+	8	34	2
+	-	3	22	1
-	+	1	0	0
-	-	1	40	18
Total		13	96	21

25

Therefore, the antigenicity of the expressed HEV peptides, E2 and GE3, was confirmed using sera from healthy donors and patients having acute viral hepatitis. Furthermore, as the results showed a markedly higher immunoreactivity from patients with acute hepatitis than in healthy donors, the expressed peptides may have antigenic properties related to the natural viral capsid proteins. In addition, it appears that E2 is the principal antigen of the two as the results also show that the number of sera reactive for E2 is substantially greater than those reactive for GE3.

35

5. HEV Specificity Determined by Two New Immunoassays

To further verify the HEV specificity of the expressed peptides, E2 and E3, three EIAs were used in which one was a commercially available test kit [Genelab Diagnostics, Singapore]. Assays were performed according to manufacturer's instructions. The other two assays were produced with purified HEV peptides, E2 and purified glutathione transferase peptide (E3). Production and purification of these HEV peptides have been previously described.

- 10 The commercial test kit was made from a mixture consisting of a 42 aa peptide and a 33 aa peptide encoded from the 3' end of ORF2 and ORF3 of a Burmese and a Mexican strain of HEV (Yarborough et al., 1991). The 33 aa peptide of ORF3 used in the commercial test is found within a portion of GE3 because, as described previously, GE3 is a 38 aa peptide which is also expressed from the 3' end of ORF3. Therefore, it is expected that the antigenic specificity of the 33 aa peptide from ORF3 in the commercial test would be closely related to that of GE3. On the other hand, the ORF2 specified peptide used in the commercial test is located beyond the carboxyl terminal end of the 215 aa E2 peptide which is disclosed in the present invention. Therefore, the antigenic specificity of the commercial test as it pertains in particular to ORF2 is likely to be distinct from E2. Unlike HEV peptides previously reported, the antigenic activity of E2 is mainly attributed to conformational changes brought about by interactions to form dimers.

- In the present study, levels of HEV specific IgG and IgM antibodies were determined by two new EIAs produced with the same purified preparations of the HEV peptides. HEV specific IgG antibodies contained in 86 of the patients' sera were additionally determined by a commercially available assay. Comparison of the results obtained by the three assays of distinct antigenic specificity permitted the assessment of antibody responses to three distinct domains of the HEV capsid. The patients' sera was obtained at different times after onset of the disease, and this proved to be especially useful, allowing the relation of serological findings to the onset of the disease to be determined.

- The two new EIAs were produced by coating microplates with purified preparations of E2 and E3, respectively (Figure 10). Purity of these preparations were estimated by

relativ intensity of the 42 kD E2 dimer and 30.4 kD E3 fusion peptide as to constitute 89% and 95% of the total proteins in their respective preparations (Figure 10, left lanes). Western blotting showed that these peptides were the principal antigens in these preparations recognized by HEV reactive human sera (Figure 10, solid and hatched circles), but not by the control negative human serum (Figure 10, open circles). Titration of these sera by assays produced with these purified HEV peptide preparations yielded typical results which correlated with reactivity of these sera against corresponding peptides as determined by Western blotting.

- 10 In an extended study, levels of HEV IgG antibodies were determined in serum specimens obtained from 90 donors and 96 patients with current, or past history of non-A, B and C acute hepatitis by E2 and E3 specific assays. Antibody levels of individual patients sera generally correlated with their reactivity against the corresponding viral peptides previously determined by Western blotting (Figure 11):
- 15 OD values obtained for the reactive sera (Figure 11, solid bars) determined by Western blotting were higher than the weakly reactive (Figure 11, hatched bars) or the non-reactive (Figure 11, open bars).

- By setting cutoff values at 3 SD above the mean OD values of non-reactive sera evidenced by Western blotting, 93% of E2 reactive or weakly reactive sera gave a positive result by E2 specific assay, and 79.4% of E3 reactive sera gave a positive result by E3 specific assay. Total concordance between Western blotting and E2 and E3 specific assays were 95.8% and 91.6%, respectively. Discrepant results obtained by these assays and Western blotting were confined to the weakly reactive sera.
- 25 These results confirmed that the E2 assay was specific for the 42 kD E2 dimer and the E3 assay was specific for the 30.4 kD E3 peptide. Seroprevalence of IgG E2 antibodies as determined by EIA was 54.2% for the patients and 11.1% for the donors, and that of IgG E3 was 29.2% for the patients and 3.3% for the donors.
- 30 Seroprevalence of E2 specific antibody of either group of test subjects was higher than the E3 specific antibody. This suggested that E2 is the dominant of the two antigens and that a higher prevalence of either of these antibodies was associated with acute hepatitis.

6. Antibody Responses to Current and Past HEV Infection

The level of E2 and E3 specific IgM antibodies of sera from patients and donors was also determined. In a preliminary study, it was shown that the assay was not affected by the presence of IgG antibodies, such that results obtained in the presence and absence of anti-human IgG were essentially the same. Subsequent determinations were therefore done in the absence of anti-human IgG and cutoff values were set at 3 SD above the mean OD values of sera from 90 donors. Figure 12 compares the occurrence of E2 and E3 antibodies in sera from patients with time of onset of hepatitis. E2 and E3 specific IgM antibodies and E3 specific IgG antibodies were mainly present in serum samples obtained early after onset of hepatitis while occurrence of E2 specific IgG antibodies were not related to disease onset.

Based on HEV antibody levels determined by E2 and E3 specific assays as in Figure 12, individual serum specimens tested exhibited 10 distinct HEV serological profiles. A total of 64 patient sera were found to variously reactive for one or more of these antibodies (Table 2). Twenty-nine sera exhibited seven serological profiles which are consistent with current infection (Table 2, profiles 1 to 7). These include 7 sera which were reactive for E2 IgM only (Table 2, profile 1), 8 sera which were additionally reactive for E2 and E3 specific IgG antibody (Table 2, profile 4) and another 8 which were reactive for these and also E3 specific IgM antibody (Table 2, profile 7). The other sera were reactive for E2 specific IgM and various other antibodies. It was noted that 25 of these samples were taken within 42 days after onset of hepatitis. The other 4 patients' sera were taken more than 60 days after onset. These and the other 3 sera from donors presumably were due to asymptomatic HEV infection unrelated to previous episodes of hepatitis. Another 35 patients' sera exhibited serological profiles, which are consistent with past HEV infection (Table 2, profiles 8 and 9) being positive for IgG antibodies but not IgM antibodies. Twenty-five of these sera were reactive for E2 specific IgG (Table 2, profile 8) and the others were also reactive for E3 specific IgG (Table 2, profile 9), but none were reactive for the IgM antibodies (Table 2, profiles 8 to 10). One profile showed sera that was not reactive to any of these antibodies (Table 2, profile 10). However, occurrence of these serological profiles were not related to time of onset of hepatitis. Most of the specimens showing current infection profiles were obtained within 27 days (median = 14 days) after onset of hepatitis and the other specimens were obtained on days indicated in parenthesis. Past infection

profiles did not correlate with disease onset, and these specimens were cumulated at a similar rate after disease onset as the non-reactive sera (median = 70 and 50 days).

Table 2
Serological Profiles of HEV Infection

Profiles Identified	IgM		IgG		Donors	Patients	Days After Onset
	E2	E3	E2	E3	No. of Sera (n=90)	No. of Sera (n=96)	
1	+	-	-	-	0	7	8 - 17 (210)
2	+	-	+	-	0	1	9
3	+	-	-	+	0	1	9
4	+	-	+	+	0	8	5 - 27 (190,240)
5	+	+	-	-	3	3	8 - 15 (60)
6	+	+	-	+	0	1	8
7	+	+	+	+	0	8	7 - 15 (40,42)
Total (Current Infection)					3	29	Median = 14
8	-	-	+	-	10	25	5 - 430
9	-	-	+	+	0	10	9 - 310
Total (Past Infection)					10	35	Median = 70
10	-	-	-	-	77	32	Median = 50

The avidity of E2 specific IgG was further studied in 16 acute sera reactive for this antibody (Table 2, profiles 4 and 7) and those present in 22 other sera which exhibited serological profiles of past infection (Table 2, profiles 8 and 9).

In Table 3, E2 specific IgG antibody levels were titrated in the presence and absence of 4 M urea as described in the section entitled "Materials and Methods". Antibody titres were defined as reciprocals of serum dilution which yielded an OD value of 1.0. The avidity index which was higher than or equal to 4, was considered to indicate a low avidity of the antibody. The cut-off value for E2 IgM was 0.40 as indicated in Figure 12. Table 3 shows that titres of 12 out of 16 acute sera were reduced by more than four times by treatment with 4 M urea, while all except one of 22 sera (Table 3, sample no. 12) which exhibited past infection profiles were not significantly affected by this treatment. Low avidity of E2 specific IgG antibody as evidenced by its susceptibility to treatment with urea suggested that such antibody was produced early after primary HEV infection. The occurrence of which was correlated with the occurrence of E2 IgM

and coincided with early onset of hepatitis.

Table 3
Avidity of E2 Specific IgG Antibody

5	Serological Profiles ¹									
	IgM		IgG		Sample No.	Days After Onset	E2 IgM (OD)	E2 IgG Titre		Avidity Index ²
	E2	E3	E2	E3				Control	Treated	
10	+	-	+	+	118	5	1.02	500	40	12.5
					127	8	0.77	120	25	4.8
					139	8	0.70	210	30	7.0
					48	13	1.13	300	70	4.3
					69	15	0.47	300	60	5.0
15					142	27	0.67	300	50	6.0
					65	190	0.60	250	76	3.3
					72	240	0.85	200	70	2.9
20	+	+	+	+	41	7	1.23	300	50	6.0
					66	7	0.72	400	50	8.0
					109	12	1.80	700	25	28.0
					37	14	1.03	200	20	10.0
					137	14	1.10	200	10	20.0
					25	15	0.60	400	30	13.3
25					101	40	0.47	230	76	3.0
					24	42	0.45	110	40	2.8
30	-	-	+	-	79	9	0.24	140	110	1.3
					63	10	0.38	80	60	1.3
					59	50	0.21	190	130	1.5
					119	70	0.26	100	70	1.4
					81	90	0.31	170	150	1.1
					85	91	0.36	180	82	2.2
					135	97	0.26	240	140	1.7
35					83	215	0.13	140	110	1.3
					27	240	0.17	140	52	2.7
					90	380	0.19	130	72	1.8
					60	380	0.23	140	110	1.3
					97	430	0.13	240	150	1.6
40	-	-	+	+	68	9	0.35	190	61	3.1
					12	17	0.26	400	70	5.7
					84	28	0.18	500	140	3.6
					78	30	0.11	140	80	1.8
45					86	40	0.24	120	50	2.4
					94	45	0.30	500	320	1.6
					74	50	0.28	120	73	1.6
					98	70	0.20	400	260	1.5
					121	200	0.34	73	60	1.2
					96	310	0.19	100	51	2.0

¹ Serological profiles as described in Table 2

² Avidity index = control titre/treated titre

7. Antibody Responses to Distinct Antigenic Domains of HEV Capsid Proteins

5 For further comparison, the level of HEV IgG antibodies was determined in 86 patients' sera by a commercially available assay (Figure 13). As evidenced by Western blotting, 32 of the sera were previously tested to be reactive for both E2 IgG and E3 IgG (E2+/E3+, Figure 13A and 13B), 14 were reactive for E2 IgG only (E2+/E3-, Figure 13 B and 13E) and 40 were not reactive against either peptides (E2-/E3-, Figure 13 C and 10 13F). The commercial assay was produced with a mixture of two peptides, one of them is similar to E3 and the other is specified by a sequence of ORF2 adjacent to the sequence that specifies E2. Consequently, levels of antibody determined by the commercial assay for the E2 and E3 reactive sera varied coordinately with levels of E3 antibody (Figure 13A) but independently with the levels of E2 specific antibody (Figure 15 13D). Seven of these sera were weakly reactive for E3 and gave low OD values which were below but close to the cut-off values of the E3 specific EIA (Figure 13A). Thirteen of the 14 sera reactive only against E2 were found to contain different levels of the corresponding antibody, 7 of which were also variously reactive by the commercial assay (Figure 13E). However, all of them gave a negative result by the E3 specific EIA 20 (Figure 13B). The remaining sera were not reactive against either peptide. All of them gave a negative result by both E2 and E3 specific EIA, and all but one also gave a negative result by the commercial assay.

The spectrum of HEV antibody detected by the three assays is summarized in Table 4. 25 Despite their distinct antigenic specificity, 37 sera were positive by both the E2 specific assay and the commercial assay and 40 were negative by both assays, giving a total concordance of 89.5% between the two assays. Similarly, total concordance between the E3 specific assay and the commercial assay was 75.6%. Overall concordance between the three assays combined was 73.3%. Frequency of detection of HEV 30 antibody by the commercial assay (47%) was slightly lower than the E2 specific assay (50%) and higher than the E3 specific assay (29%). Based on the antibody spectrum exhibited by individual sera, antibody detected by the commercial assay in 15 of 38 sera can be attributed to those which are specific for the ORF2 specified peptide used to produce this assay because the sera were not reactive when tested by the E3 35 specific assay. The remaining 23 sera gave a positive result by both the commercial

assay and the E3 specific assay, but it was not ascertained if these sera also contained antibody against the ORF2 specified peptide used to produce the commercial assay.

5

Table 4
Viral Hepatitis Patients' Sera Reaction Patterns
Against HEV Peptides and Commercial EIA Kit

10	Western Blotting (IgG)		Commercial EIA Kit	
	E2	GE3	IgG	Number of Sera (n=86)
	+	+	+	23
	+	-	+	14
	+	-	-	6
	-	+	+	2
15	-	-	+	1
	-	-	-	40
	Prevalence (%)	50	29	47
	Total Concordance (%) ¹	89.5	75.6	-

20

1 Total concordance (%) with the commercial assay equals the number of sera giving a positive result or a negative result by either E2 or E3 specific assays and the commercial assay simultaneously/total number of sera tested x 100%. Overall concordance between the three assays combined similarly was calculated to be 73.3%.

25

(D) Establishment of ELISA to Detect IgG and IgM Anti-HEV

1. IgG and IgM Anti-E2 Detected by ELISA

30

The expressed HEV peptide E2 was used to develop two ELISA tests for the detection of IgG and IgM anti-HEV in human serum. The results of the IgG ELISA indicated that IgG anti-E2 was detectable in most patients who had confirmed current or previous infection by HEV. Such antibodies were rarely detected in healthy blood donors. In an additional study, the level of IgM anti-E2 was similarly determined using the ELISA. IgM antibodies are often detected in the serum initially after an infection, the level of which can provide a diagnosis of acute infection with HEV.

35

Th IgG and IgM anti-E2 levels of 96 sera from patients was tested by ELISA from 5 to 430 days after the onset of acute hepatitis. OD-TIME graphs were derived according to the OD value and the day of the sample. Figure 12B shows that IgM antibody against E2 was detected at a very early state of the infection, mainly within 2 weeks following the onset of HEV. Alternatively, Figure 12A shows that while IgG anti-E2 was elicited shortly after IgM anti-E2, it persists in the sera for more than 1 year.

2. IgG Anti-E2 Detected by Western Blot

10 All the sera used in the previous example was retested by Western blot. The results confirmed that most of the antibody determined by EIA could be attributed to those which could bind to the dimeric form of E2, but not its monomeric form.

15 The results of the Western blot were compared to those results derived using EIA for the detection of IgG (Table 5). The concordance of the two tests is 92.7% which confirms that most of the antibodies detected by EIA could be attributed to those which bind only to the dimeric form of E2.

Table 5
Comparison of Western Blot and EIA for IgG Anti-E2 and Anti-E3

E2 Assay	<u>Western Blot</u>		Total
	Positive	Negative	
Positive	52	0	52 (52.2%)
Negative	4	40	44
Total	56 (58.3%)	40	96

E3 Assay	<u>Western Blot</u>		Total
	Positive	Negative	
Positive	27	1	28 (29.2%)
Negative	7	61	68
Total	34 (35.4%)	62	96

- 15 E2 and E3 specific IgG antibodies were determined by EIA and Western blotting at 1:100 and 1:250 serum dilution, respectively. Cut-off values are as shown in Figure 12.

(E) Establishment of Immune Capture RT-PCR to Detect HEV RNA

1. Specificity of RT-PCR Primers

In this study, two pairs of primers, A5/A3 and B5/B3 (Table 9) were chosen for RT-PCR. The specificity of these primers was evaluated by direct RT-PCR with specimens separately containing HEV, HAV, enteroviruses and caliciviruses. Figure 14 shows that only the specimen containing HEV presented a specific band with the expected size of 203 bp.

2. Immune Capture RT-PCR (IC-RT-PCR)

Antiserum raised against E2 in rabbits was tested for its ability to immune capture HEV. Polystyrene paddles, coated separately with the antiserum and the pre-immune

serum, were used for capture of the virus. RT-PCR on the captured particles was performed as described in the section entitled "Materials and Methods" in this disclosure. It was found that the antiserum could capture HEV particles up to a dilution of 5⁵ of the stock virus, while the pre-immune serum failed to capture the virus even
 5 with undiluted stock solution (Figure 15).

3. Application of IC-RT-PCR

Thirteen stool specimens from confirmed acute hepatitis E patients and 45 stool
 10 specimens from experimentally-infected monkeys were tested by IC-RT-PCR. It was found that six of the former and 18 of the latter were positive. The positive specimens from the infected monkeys were taken between day 5 and day 19 after the HEV challenge.

15 With public health and environmental monitoring, 64 shellfish samples collected from street markets and 17 from three estuaries in Hong Kong have been tested using IC-RT-PCR. HEV was detected in two of the former and three of the latter.

The lack of sera from hepatitis E patients had previously limited the application of the
 20 IC method. However, the IC-RT-PCR method described above can be used routinely for the detection of HEV for public health and environmental monitoring because of the availability of a stable source of antisera derived from the expression of HEV specified protein.

25 In our method, IC was followed by the extraction of HEV RNA with a commercial kit (QIAamp Viral RNA Kit, QIAGEN) the use of which is familiar to molecular biologists. If it is assumed that the efficiency of RNA extraction using a commercial kit is 100% without interference, then theoretically, IC-RT-PCR should be 32 times more sensitive than direct RT-PCR because the former can accommodate 32 times more volume of
 30 sample. In this study, the seeding virus was diluted in 5-fold serial dilution so the expected results should be that IC-RT-PCR is 25-fold more sensitive than RT-PCR. The results (Table 6) indicate that HEV in water is relatively more stable than predicted. If HEV is not stable in 4°C for overnight, the HEV will decay dramatically and therefore, the sensitivity of IC-RT-PCR will not be as high as it is.

35

To stool and shellfish specimens, the method has a lower sensitivity than for water. This phenomenon may be caused by some unknown factors which can accelerate the degradation of viruses. On the other hand, the IC-RT-PCR results are 125-fold more sensitive than direct RT-PCR which implies that IC successfully overcame the
5 interference from specimens which may reduce the efficiency of the RNA extraction kit.

IC-RT-PCR is practical as a clinical and environmental monitor. When the method was applied to patients' stool and shellfish collected from markets and estuaries, HEV was detected in 46% stool specimens of hepatitis E patients confirmed by the serological
10 method. Accordingly, 3% of shellfish in market and 17.6% shellfish collected from estuaries were HEV RNA positive. These results show that IC-RT-PCR is a feasible method for the detection of HEV RNA.

4. Comparison of a Commercial Viral RNA Detection Kit to IC-RT-PCR

15 Three types of HEV-seeded specimens were prepared. These included plain water, the supernatant of human stool specimens and the supernatant of homogenized shellfish specimens. The comparative efficiency of HEV capture from these specimens was examined (Table 6). The results show that IC-RT-PCR is at least 25 times more
20 sensitive than the commercial viral RNA kit and that there are unknown factors in the supernatant of stool and shellfish which inhibit the extraction of RNA for subsequent synthesis of cDNA. However, interference is reduced to a minimum using IC-RT-PCR.

25 **Table 6**
Comparative Efficiency of HEV Detection by RT-PCR

Method	Water	Stool Supernatant	Shellfish Supernatant
IC-RT-PCR	55	53	53
Viral RNA Kit	53	50	50

(F) Establishment of Sandwich ELISA for the Detection of HEV Antigens

Rabbit anti-GE2 antibodies were used to coat microtitre plates, and HRP conjugated

monkey anti-E2 IgG was used as the detector (see "Materials and Methods"). This method was used to detect HEV antigens in stool specimens of monkeys which were experimentally infected with HEV. The cut-off value was set at 3 SD above mean OD values of pre-challenged stool samples. Seventeen out of 45 stool specimens were positive for the detection of HEV antigen from day 1 to 21.

(G) The Role of E2 in HEV Protection

The previous results of the study of non-A, B and C hepatitis patients, indicated that the dimeric form of the recombinant peptide, E2, may assume an important role in natural HEV infection through the exposure of conformational antigenic determinants which are generated from the dimerization of the monomeric form of the peptide.

As previously shown in the study of non-A to C acute hepatitis patients, the dimeric form of E2 was found to assume a more prominent role in natural HEV infection. E2 specific IgM antibodies were commonly produced during acute HEV infection. The corresponding IgG antibodies were also produced and persisted for a protracted period of time accompanied by increasing avidity. Furthermore, they were the most prevalent HEV antibodies present in convalescent sera and sera from individuals previously infected with the virus. These results suggested that E2 may afford protection against HEV and is supported by a protection study in the experimental infection of Macaque monkeys. Moreover, the results also suggest that the protective effects are mainly attributed to conformational antigenic determinants, rather than linear epitopes and provides a rational basis for vaccine development.

A protection study in experimental infection using a Macaque monkey model shows that immunization with a purified preparation of the peptide confers protection of the animals against a HEV challenge and therefore, makes it a prime vaccine candidate. Virus excretion in stool and viraemia seen in the control animals were essentially abrogated in the immunized animals. None of these animals developed additional HEV antibodies apart from E2 antibodies already present before the challenge. Since E2 antibodies present in the pre-challenge sera were predominated by those which specifically recognize the E2 dimer rather than its monomeric form, it was concluded that to a large extent, the protective effects are attributed to the conformational

antigenic determinants arising from interactions with this viral peptide. Consistent with this belief, previous studies suggest that E2 probably encompasses a domain in the major structural protein of HEV, which interacts to form HEV capsid. Therefore, the viral capsid is generated with the same or similar conformational antigenic

5 determinants compared to those generated through the E2 dimerization.

Consequently, antibodies specific for E2 dimers are the dominant antibody response to natural HEV infections (see Figure 16B) and these antibodies were also developed in control animal 5 following a HEV challenge.

10 1. Immunization

Three monkeys were immunized with four weekly intra-muscular doses containing ___ mg of purified E2 and the animals were bled one week after the final dose. The

15 reported nucleotide sequence and predicted amino acid sequences in the

corresponding regions of prototype HEV strains. The 213 aa peptide is encoded in a highly conserved region in the ORF2 of the HEV genome (Table 7). Purity of the viral peptide used for immunization was assessed by PAGE (Figure 16A) and its

20 antigenicity, by immune blotting using a human HEV reactive and a non-HEV reactive serum (Figure 16B). The viral peptide was heated for 10 minutes at 100°C. The

denatured peptide was then mixed in equal proportions with unheated native peptide before electrophoresis. In agreement with previous findings, the native peptide was resolved as a 42 kD dimer and dissociated into a 26 kD monomer after heating. These two peptides combined constituted over 90% of the protein present in the purified

25 preparation used for immunization. The dimeric form was specifically recognized by the HEV reactive human serum (Figure 16B, Lane 1) but not by the non-HEV reactive serum (Figure 16B, Lane 2). The 26 kD monomeric form, however, was not recognized by the HEV reactive serum although previous peptide mapping studies

30 predicted that E2 may contain an array of linear antigenic epitopes. This suggests that HEV antibodies present in the human serum is mainly directed against conformational antigenic determinants arising from interactions between the viral peptide.

Table 7
A Conserved Region of the HEV Major Structural Protein

5	Strain	Region	Sequence Homology (%)		Reference
			Nucleotide	Amino Acid	
	D11092	China	100	100	Aye et al., 1992
	D10330	China	92.6	98.6	Aye et al., 1993
	L25547	China	94.1	99.5	Yin et al., 1994
10	M73218	Burma	92.9	100	Tam et al., 1991
	M74506	Mexican	78.2	94.3	Purdy et al., 1999
	M80581	Pakistani	98.3	100	Tsarev et al., 1992
	M94177	China	98.6	99.5	Bi et al., 1994
	X98292	India	89.7	99	Donati et al., 1997

HEV antibodies produced by the animals in response to immunization was titrated by immune blotting against the same mixture of native and heat denatured E2 (Figure 17A, Lanes 1 to 4). The sera were further tested by immune blotting against purified E3 (Figure 17B), a GST fusion peptide specified by a HEV specific sequence located at the 3' terminus of ORF3 of the viral genome and by a commercial EIA assay (Figure 17C). According to the manufacturer, the assay was produced with two HEV peptides, one is similar to E3 and the other corresponds to the C terminal region of the major structural protein of HEV which is located adjacent to, but does not overlap with E2.

E2 dimer specific antibody titres of the post-immunization sera were 1:64,000 for animal 1, 1:1,600 for animal 2 and 1:4,000 for animal 3 (Figure 17A). None of the sera obtained from these animals before immunization showed detectable activity at 1:100 serum dilution (Figure 17A, Lane 5). Sera from animals 1 and 3, but not animal 2, were additionally reactive against E2 monomer however titres of these antibodies were 16 and 4 times lower than the corresponding level of antibodies specific for the E2 dimer. None of these sera obtained before or after immunization showed reactivity against E3 in the HEV minor structural protein nor the domain located adjacent to E2 in the HEV major structural protein (Figure 17B and 17C). It was concluded from these results that immunization had elicited a specific antibody response against the conformational antigenic determinant generated by the dimerization of E2.

2. HEV Challenge

The immunized animals and three control animals were challenged 2 weeks after

immunization by intra-peritoneal injection of a strain of HEV. The challenging virus was originally isolated from an outbreak in China in 19__ (see also Table 7) and the amount of the virus injected was estimated by PCR to be _____ HEV genome equivalent per dose (Figure 18). The primate infection dose of the challenging virus injected was not
5 determined. Recent studies by other investigators showed that primate infectivity titres of HEV are of the same order of magnitude as genomic titres. The animals were monitored every 2 days for virus excretion in stool and peripheral blood was collected every week from the animals after the virus challenge for 6 weeks. Peripheral blood specimens were obtained for determination of liver enzymes, viraemia and HEV
10 antibody responses during the same period.

The large virus dose injected did not cause overt hepatitis in these animals, however, their ALT levels fluctuated within normal limits and all animals remained well throughout the observation period. Nevertheless, HEV excretion was persistently
15 detected for 10 to 12 days in stool samples obtained from all three control animals between 5 and 17 days after the challenge (Table 8). The virus was additionally detected in peripheral blood monocytes in a sample obtained 14 days after a challenge from animal 5 and in another sample from animal 7, 7 days after the challenge. However, the virus was not detected in plasma samples from these animals. Infection
20 of animal 5 was accompanied by HEV seroconversion (Figure 18). Antibody specific for E2 dimer was first detected 7 days after the challenge and also in the subsequent plasma specimens. These specimens were also reactive against E3 and gave a positive result by the commercial assay. The broad spectrum of HEV antibodies detected in these specimens was in contrast to restricted specificity spectrum seen
25 earlier in sera obtained from the test animals after E2 immunization. Infection of the other control animals was presumably milder and did not elicit a detectable antibody response.

Table 8
HEV Excretion in Stool and Viraemia After Virus Challenge

5	Group	Monkey	Day After Challenge									
			3	5	7	9	11	13	15	17	19	20
10	Test	No. 1	-	-	-	-	-	-	-	-	-	-
		No. 2	-	-	-	-	-	-	-	-	-	-
		No. 3	-	+	-	-	-	-	-	-	-	-
	Control	No. 5	-	+	+	+	+	+ ¹	+	+	-	-
		No. 7	-	-	+	+ ¹	+	+	+	+	-	-
		No. 8	-	-	+	+	+	+	+	+	-	-
15												

¹ The HEV genome was detected in peripheral blood monocytes by RT-PCR. None of the plasma samples contained detectable HEV genome.

20 Viral activity was markedly reduced in the immunized animals. Virus excretion was detected in a stool specimen obtained 5 days after a challenge from animal 3 (Table 8). Apart from this, none of these immunized animals showed detectable viral activity in stool, plasma or PBMC samples. All these animals exhibited strong seroreactivity specific for E2 dimer due to previous immunization and these antibodies were

25 sustained at high levels in all subsequent specimens obtained after the challenge, but none developed the other HEV antibodies. Compared with the control animals, these results showed that E2 may afford protection against mild HEV infection.

30 MATERIALS AND METHOD

Example 1 Cloning of HEV Capsid Gene

35 A. Extraction of HEV RNA

Viral RNA was extracted from the bile of an experimentally HEV-infected macaque monkey (Zhuang et al., 1992) using the QIAamp Virus RNA Kit [QIAGEN GbmH, Hilden, Germany] according to the manufacturer's instructions. The purified RNA was

40 mixed with 2 volumes of isopropanol [BDH Laboratory Supplies, England] and 1/10th

volum 3M NaAc (pH 6.4) [Sigma, USA] and left to stand at -20°C for 1 hour. Afterwards, the mixture was centrifuged at 15,000 rpm for 15 minutes. The pellet was washed once with 70% ethanol [BDH Laboratory Supplies, England] and then resuspended in reverse transcription buffer.

5

B. RT-PCR of Target Sequences

For reverse transcription, the RNA-pellet was added to 20 µl of RT master mixture (4 µl 5xRT buffer [Boeringer Mannheim]; 1.6 µl of 2.5 mM dNTP; 0.2 µl Avian Myeloblastosis Virus (AMV) [Boeringer Mannheim]; 0.625 µl RNAsin [Boeringer Mannheim]; 1 µl reverse primer E5R or E3R (150 µg/µl); 12.6 µl RNase-free water) and incubated at 42°C for 1 hour.

The primers used are listed in Table 9. E3R and E5R were designed to construct cDNA fragments of the virus. The cDNA sequences corresponding to the 3' terminal regions of ORF2 and ORF3 were amplified using the primer pairs ORF2F/ORF2R and ORF3F/ORF3R, respectively. These primers were modified to contain BamHI and EcoRI restriction sites to facilitate cloning of the amplified cDNA fragments. All primers were synthesized by Life Technologies, U.S.A.

20

Table 9
RT and PCR Primers

Primer	Purpose	Position	Sequence	Enzyme Site
E3R	RT	5508-5529	5'-cggggagtcacacatcaggcact-3'	
E5R	RT	7117-7140	5'-aagcaataaactataactcccga-3'	
ORF2F	Cloning	6326-6350	5'- <u>gctggatccc</u> agctgttctactctcgtcccgtcg-3'	BamHI
ORF2R	Cloning	7117-7136	5'- <u>ggcgaattc</u> caataaactataactcccga-3'	EcoRI
ORF3F	Cloning	5364-5384	5'- <u>ccgggatcc</u> gac ctc gtg ttc gcc aac ccg-3'	BamHI
ORF3R	Cloning	5457-5477	5'- <u>cagggaattc</u> ttagcggcgcgggccccagctg-3'	EcoRI
A3	Detection	4566-4586	5'-ggctcaccggagtggttcttc-3'	
A5	Detection	4341-4362	5'-cttgatgacaccgtcttctcg-3'	
B3	Detection	4554-4575	5'-gtgtttcttccaaaccctcgc-3'	
B5	Detection	4372-4392	5'-gccgcagcaaaggcatccatg-3'	

PCR amplification of the target sequence cDNA was carried out by adding 5 μ l of cDNA to 45 μ l PCR master mixture (5 μ l 10xTaq buffer) [Boeringer Mannheim]; 4 μ l of 2.5 mM dNTP mixture; 1.0 μ l of each primer (150 ng/ μ l); 1 μ l of Taq DNA polymerase (1 U/ μ l) [Boeringer Mannheim] and 33 μ l ultrapure water. The PCR mixture was overlaid with 50 μ l mineral oil. The thermal cycling conditions were: denaturation at 94°C for 40 seconds; annealing at 57°C for 40 seconds; and extension at 72°C for 1 minute. The resulting PCR products were designated e2 and e3 from ORF2 and ORF3, respectively.

C. Cloning into pGEX Plasmid

The PCR products were extracted with phenol-chloroform and then precipitated with ethanol. The products, e2 and e3, and the vector pGEX₂₀ were digested using BamHI and EcoRI (Boeringer Mannheim). The pGEX₂₀ vector was a gift from Dr. Cao Liang, Department of Microbiology, the University of Hong Kong. It was a derivative of a pGEX expression vector (Smith et al., 1988) with the multiple cloning

site 5'-CCGCGTGGATCCGAAATTCCTCGAGATCGATTAG-3' containing BamHI, EcoRI, XhoI and ClaI restriction cleavage recognition sequences. The digested fragments were separated on agarose gels, recovered by cutting the band out of the gel, electro-elution and then precipitated by ethanol. Afterwards, e2 and e3 were

5 ligated to pGEX₂₀ using T4 ligase (Boehringer Mannheim). The recombinant plasmids pGEX₂₀-e2 and pGEX₂₀-e3 were transformed into E.Coli DH5 α by electro-transformation with a gene pulser [BIO-RAD] and plated on LB agar plates (Sambrook et al., 1989) with ampicillin (100 μ g/ml). Twenty colonies of transformants were picked up for plasmid preparation. BamHI and EcoRI digestion was subsequently carried out

10 and recombinants with the expected insert size were chosen. All plasmids used in this study were prepared using the QIAgen mini-plasmid kit [QIAgen, Hilden, Germany].

D. Sequencing

15 DNA sequencing was performed using an ABI PRISM Dye Terminator Cycle Sequencing Ready Reaction Kit [PERKIN ELMER]. The results showed that the 811 bp e2 sequence was located at position 6326-7136 and also revealed a single base pair deletion at position 6957, presumably due to a PCR amplification error. The resulting frameshift was predicted to cause translation to terminate prematurely at a

20 new stop codon at position 6968 giving a smaller than expected peptide of 213 aa with a MW of 23 kD, instead of 267 aa as initially expected. The position of e2 and relative fragments are shown in Figure 1.

Example 2

25 **Production and Purification of HEV Peptides**

A. Expression of GST Fusion Protein

The recombinant plasmids were transformed into E.Coli BL21. Single colonies were

30 picked for growth in 2xYTA medium (tryptone 16 g/l; yeast extract 10 g/l; NaCl 5 g/l; ampicillin 100 μ g/l). The overnight culture (4 ml) was inoculated in 400 ml of 2xYTA medium and incubated at 28°C until the OD₆₀₀ was ≥ 0.5 . Isopropyl β -D-Thiogalactoside (IPTG) [Pharmacia Biotech, U.S.A.] (400 μ l of 100 mM solution) was added and the culture grown for 5 to 6 hours. The cells were pelleted by centrifugation

at 7,000 rpm for 10 minutes in a Beckman J2-MC rotor JA-14.

B. Purification

- 5 The pellet was washed once in phosphate buffer saline (PBS: 0.8% NaCl; 0.02% KCl; 0.144% Na₂HPO₄; 0.024% KH₂PO₄, pH 7.0), resuspended in 20 ml PBS and sonicated in a SONIPREP 150 [MSE] (30 seconds on; 30 seconds off; 35 cycles; power 18 to 22). After sonication, Triton-X100 (Sigma, U.S.A.) was added to give a final concentration of 1% and the mixture was gently shaken for 30 minutes. The bacterial
- 10 lysate was centrifuged at 4°C and the supernatant was collected. Batch purification of the fusion protein was carried out according to the "GST Fusion Protein System Manual" using glutathione sepharose-4B [Pharmacia Biotech, U.S.A.]. The bound fusion peptide was eluted twice with elution buffer (10 mM reduced glutathione in 50 mM Tris-HCl, pH 8.0) and are referred to as GE2 and GE3 to correspond to the
- 15 cDNA fragments of ORF2 and ORF3, respectively.

C. Thrombin Cleavage of Fusion Proteins Bound to Bulk Matrix

- Thrombin [Pharmacia Biotech, U.S.A.] (5 µl of a 1 U/µl solution) and 95 µl of PBS
- 20 were added to a 100µl bed volume of GE2 bound to glutathione sepharose-4B and incubated at 22° for 16 hours. The supernatant was collected by centrifugation and pooled with the supernatant of a second wash of the matrix. This thrombin-cleaved protein was designated E2.

25

Example 3

Identification of HEV Peptides

A. Analysis of Polyacrylamide Gel Electrophoresis (PAGE)

- 30 A 10% of SDS-polyacrylamide gel was set according to standard methods (Sambrook et al., 1989). The peptide specimens (4µg) were loaded on the gel and electrophoresed at 100 volts for 3 hours using Minigel Twin G42 [Biometra, Germany]. The gel was stained with Coomassie brilliant blue R250 in a mixture of 45 ml methanol, 45 ml H₂O and 10 ml of glacial acetic acid.

B. Western Blotting

After SDS-PAGE, the proteins in the gel were transferred to 0.45 μ m nitrocellulose membranes [BIO-RAD, U.S.A.] at 100 volts for 1 hour using Mini-PROTEAN II Cell [BIO-RAD]. After being blocked with 5% of skim milk [Carnation, Nestle] in 1xPBS at 4°C overnight, the membrane was reacted with pooled sera (1:500) collected from hepatitis E patients or anti-GST sera (1:1000) obtained from guinea pigs at room temperature for 1.5 hours. After washing three times, 5 minutes each time with 0.05% Tween₂₀ in PBS, the membrane was reacted with horseradish peroxidase (HRP) conjugated Protein-A [BIO-RAD, U.S.A.] at room temperature for another 1.5 hours. After washing three times again, the positive bands were developed by incubation of the membrane with 3-amino-9-ethyl carbazole [AEC Single Solution, ZYMED, U.S.A.] at room temperature for 5 to 10 minutes. The colour reaction was stopped by transferring the membrane into water. The sera and conjugates described above were diluted in blocking buffer (5% of skim milk [Carnation, Nestle] in 1xPBS).

Example 4

Establishment of ELISA to Detect IgG and IgM Anti-HEV

20 A. Serum Source

The sera from 96 non-A, B and C hepatitis patients collected at the Princess Margaret Hospital, Hong Kong, was studied. Of these, 86 out of 96 were further tested with a commercial HEV kit [Genelabs, Singapore]. Among the 86, 45 were IgG anti-HEV positive and 15 were IgM anti-HEV positive. Sera from 90 healthy donors was obtained at the Queen Mary Hospital.

B. ELISA Using HEV Peptide Antigen

30 Polystyrene microtitre plates [Nunc, Denmark] were coated with 100 μ l of purified peptide E2 (6.5 ng/100 μ l in 0.05 M sodium carbonate, pH 9.5) in each well. After overnight incubation at 4°C, the wells were washed with 350 μ l washing buffer (0.05% Tween₂₀ in PBS) and then blocked with 2% bovine serum albumin (BSA) [Sigma, U.S.A.] in PBS at 4°C for 24 hours. The plates were rewashed twice again with

washing buffer. 100 μ l of 1:100 diluted serum (diluent: 1% BSA, 0.2% Bronidox in PBS) was added to each well. After 30 minutes incubation at 37°C, the plates were washed five times. Then HRP-conjugated goat anti-human IgM or HRP-conjugated protein A (diluent: 1% BSA, 0.2% bronidox, 10% sucrose in PBS) is added to each well and incubated at 37°C for 30 minutes. After five washings, 100 μ l of 3,3', 5,5'-tetramethylbenzidine (TMB) [Diesse, Italy] was added to each well and incubated at 37°C for 15 minutes. The reaction was then stopped by adding 0.3 M H₂SO₄ to each well. The plate was read at an absorbance of 450 nm. Anti-E2 IgG cut-off values were set at 3 SD above the mean OD value of non-reactive sera previously tested by Wester blot. The anti-E2 IgM cut-off value was set at 3 SD above the mean OD value of the healthy blood donors' sera.

The assay specific for E2 was produced by coating the microplates with 0.063 μ g/ml of the peptide and assay specific for E3 by coating microplates with 0.23 μ g/ml of GE3. Concentrations of the peptides used were previously determined to be optimal. HEV antibody levels were determined by adding 0.1 ml serum specimens at 1:100 dilution to duplicate wells. After incubation at 37 °C for 30 minutes, the wells were washed five times with 0.05% Tween₂₀ in PBS. IgM antibodies were determined by reaction with a horseradish peroxidase (HRP)-conjugated human IgM specific antiserum at 1:25,000 dilution [BIOSOURC] and IgG antibodies with a HRP-conjugated protein A at 1:16,000 dilution [BIO-RAD]. After incubation at 37 °C for 30 minutes, the wells were washed five times and 100 μ l TMB substrate (3,3',5,5'-tetramethylbenzidine) was added. The reaction was stopped by 0.3 M H₂SO₄ after incubation at 37 °C for 15 minutes. The plate was read at 450 nm with an Anthos 2001 microplate reader [ANTHOS LAB]. To enable comparison of the results obtained on separate test runs, a reference serum was included in each test run, and OD values obtained with test sera on each test run was normalized against that obtained concurrently with the reference sera.

C. IgG Avidity Test

The sera was serially diluted and reacted with E2-coated microplates in duplicate wells. After incubation at 37°C for 30 minutes, the wells were washed and then treated with

PBS (control) or PBS containing 4 M urea at room temperature for 10 minutes. The plate was washed and then reacted with HRP-Protein A conjugate as before.

Example 5

5

IgG Anti-E2 Detected by Western Blot

A. Serum Source

All the sera used in Example 4 was retested by Western blot.

10

B. Western Blot

Thirty-four (34) μ g of purified E2 was loaded to a 70 mm wide single lane SDS-polyacrylamide gel and electrophoresed at 100 volts for 3 hours. After electrophoresis, the peptides were transferred to a 0.45 μ m pure nitrocellulose membrane [BIO-RAD] at 100 volts for 1 hour in a MINI-PROTEAN II Cell [BIO-RAD]. After shaking in blocking buffer at 4°C overnight, the membrane was cut into 2 mm strips. Strips were incubated with each of the sera separately at 1:250 dilution for 1.5 hours. They were subjected to three 5 minute washings in washing buffer and then incubated with goat anti-human IgG alkaline phosphatase conjugate at 1:30000 [Sigma, U.S.A.] at room temperature for 1.5 hours. After three washings, BCIP/NBT mixture [Gibco BRL, U.S.A.] was added for colour development and the reaction was stopped by putting the strips into water.

25

All the sera used in the previous example was retested by Western blot. The results confirmed that most of the antibody determined by ELISA could be attributed to those which could bind to the dimeric form of E2, but not its monomeric form.

Example 6

30

Immune Capture RT-PCR (IC-RT-PCR)

A. Production of Specific Rabbit Polyclonal Antibodies

The antigen used for production of antibodies to HEV is fusion protein GE2. Female

white rabbits weighing 2.5-3.0 kg were immunized with 500 μ g of antigen. The first dose contained an equal volume of complete Freund adjuvant. Incomplete Freund adjuvant was used in subsequent doses in a 10 to 14 day interval schedule. When specific antibodies rose to a level detectable by EIA at 1:10000 dilution of the rabbit serum, the rabbit was given an intravenous booster of 500 μ g of the antigen in PBS. On the 4th day after the booster, blood was collected by cardiac puncture. Specific antibodies were evaluated by Western blotting and ELISA.

B. Preparation of Stool Suspensions

10

Thirteen acute hepatitis E patient's stool was collected at the First People's Hospital of Guangzhou. Stools of three experimentally HEV-infected monkeys were collected from day 0 to day 30 after HEV inoculation.

15 Stool specimens (5 g) were mixed with 20 ml 1xPBS and incubated at 4°C for 1 hour. The mixture was centrifuged at 1500 rpm for 10 minutes and the supernatant was collected for immune capture.

C. Preparation of Homogenized Suspension of Shellfish

20

Sixty-four shellfish specimens were collected from street markets around Hong Kong and 17 were collected by the Department of Environmental Protection.

25 Twenty grams of shellfish meat was blended thoroughly and the homogenate was mixed with 100 ml 0.2 M glycine-0.15 M NaCl buffer (pH 9.5) and 2 ml of stock solution of Cat-Floc (1% w/v). The resulting mixture was vortexed and incubated for 10 minutes at 4°C. The mixture was then centrifuged at 1000 rpm for 5 minutes. Supernatant was collected for immune capture.

30 D. HEV Particles Seeding

The bile containing HEV particles was collected from a confirmed experimentally HEV-infected macaque monkey generously donated by Professor Zhuang Hui (Beijing Medical University). The bile was diluted 200 fold with 1% BSA in PBS and this was

referred to as stock solution. The stock solution was diluted from 5^0 to 5^6 with five serial dilutions and these were referred to as working solution. A $10\ \mu\text{l}$ aliquot of each of the working solutions was mixed with 5 ml of either water, stool supernatant or shellfish supernatant to produce HEV-seeded specimens for IC-RT-PCR and direct RT-PCR comparison.

E. Immune Capture

Antiserum, diluted 1:100 with 50 mM sodium carbonate/sodium bicarbonate buffer (pH 9.6), was used as a coating solution. Nunc-Immuno paddle [Nunc, Denmark] was coated by incubation with 1 ml of the coating solution in a tube at 37°C for 4 hours. The antisera was removed and replaced by 1 ml of 2% bovine serum albumin (BSA) in PBS in which the paddle was incubated at 37°C for 1 hour. The paddle was then washed with 0.05% Tween₂₀ in PBS and transferred into a tube for immune capture. Shellfish suspension or 20% stool suspension (4.5 ml) was added to the tube and the tube was gently shaken overnight at 4°C . The paddle, referred to as immuno-paddle, was then washed 3 times with 0.05% Tween₂₀ in PBS and placed in a clean tube for extraction of RNA.

F. Extraction of Viral RNA

RNAse-free water ($140\ \mu\text{l}$) and AVL buffer ($560\ \mu\text{l}$) was added to the tube containing the immuno-paddle. The viral RNA was then extracted according to the instructions of the "QIAgen Viral RNA Handbook". Afterwards, 1/10 volume 2 M sodium acetate (pH 4.6) and 1 volume isopropanol was added to the purified RNA. The mixture was vortexed, left to stand at -20°C for 1 hour and then centrifuged at 14,000 rpm at 4°C for 15 minutes. The pellet was rinsed once with pre-cooled 70% ethanol. After centrifugation, the ethanol was carefully removed and the pellet was air-dried at room temperature for 15 minutes before it was reverse transcribed to specific cDNA.

G. Reverse Transcription (RT) and Nest PCR

Primers used for RT and PCR are listed in Table 9.

- 5 RT-PCR: Each RNA pellet was mixed with 20 μl of RT master mixture (4 μl 5xRT buffer [Boeringer Mannheim], 1.6 μl 2.5 mM dNTP, 0.2 μl of 25 U/ μl Avian Myeloblastosis Virus (AMV) [Boeringer Mannheim], 0.625 μl of 40 U/ μl RNasin [Boeringer Mannheim], 1 μl of 150 ng/ μl reverse primer (A3) and 12.6 μl RNase-free water). After 1 hour incubation at 42°C, cDNA (5 μl) was added to 45 μl PCR master mixture (5 μl 10xTaq buffer [Boeringer Mannheim], 4 μl of 2.5 mM dNTP mixture, 1.0 μl of forward and reverse primer (150 ng/ μl) (A3 and A5), 1 μl of 1 U/ μl Taq DNA polymerase (1 U/ μl) [Boeringer Mannheim] and 33 μl ultrapure water). The PCR mixture was overlaid with 50 μl mineral oil. The amplification was carried out in a DNA thermal cycler 480 (Perkin-Elmer Cetus) with the following cycling conditions:
- 10
- 15 denaturation at 94°C for 40 seconds; annealing at 57°C for 40 seconds; and extension at 72°C for 1 minute 20 seconds. Processing was carried out for a total of 35 cycles followed by a final auto-extension at 72°C.

- Nested PCR and amplicon detection: 2 μl of the first PCR production was added to 20 48 μl of PCR master mixture which contained the same composition as the first PCR except that the primers were B3 and B5. 5 μl of the PCR product and a 50 bp DNA ladder [GibcoGRL] were loaded to 2% agarose gel in TBE buffer and electrophoresed at 100 volts for 30 minutes. The gel was stained with 0.5 $\mu\text{g}/\text{ml}$ ethidium bromide for 15 minutes and then visualized under UV light.

25

Example 7

Detection of HEV Antigens by ELISA

A. Production of Monkey Anti-E2

30

Monkeys were immunized with 100 μg of E2 over a 2-week period. The first dose contained equal volumes of complete freund adjuvant. Incomplete freund adjuvant was used in subsequent doses. When specific antibodies were elicited to a satisfactory level, the animals were given an I/V booster. Blood was collected on the

fourth day after the booster.

B. Conjugation of Monkey Anti-E2

- 5 10 mg of horseradish peroxidase (HRP) [Type VI, Sigma] was dissolved in 1 ml of 0.1 M phosphate buffer (pH 6.8) with 1.25% glutaraldehyde [Grade U, Sigma] and incubated at room temperature for 18 hours. The mixture was then dialysed against 4 litres of 0.15 M NaCl for 24 hours at 4°C with 6 changes of solution. Polyclonal monkey IgG was purified by ammonium sulphate precipitation (40-50%) and
- 10 resuspended in a minimum volume of 0.15 M NaCl, then dialysed against the same solution to remove residual ammonium sulphate. 5 mg of the purified IgG in 1 ml of 0.15 M NaCl and 0.1 ml 1 M carbonate buffer (pH 9.6) were added to 1 ml of the dialysed HRP and the mixture was incubated at 4°C for 24 hours. A 0.1 ml of 0.2 M lysine in 0.15 M NaCl was added and the mixture was left to stand at 4°C for 24 hours
- 15 to block unconjugated sites. The mixture was then dialysed against PBS for at least 24 hours. The conjugated monkey IgG was precipitated with an equal volume of saturated ammonium sulphate and then resuspended in a minimum volume of PBS and dialysed against PBS for 24-48 hours as described above. The mixture was centrifuged at 13,000 rpm for 20 minutes and the pellet discarded. BSA was added to
- 20 the supernatant to 1% and thimersol was added to 0.01%. The preparation was tested by direct E2 ELISA before it was stored at -70°C.

C. Antibody Sandwich ELISA to Detect HEV Particles

- 25 Rabbit anti-GST-E2 was diluted in 0.05 M carbonate buffer (pH 9.5). 100 µl of diluted serum was added to each well of a microtitre plate and incubated at 4°C overnight. After two washings with washing buffer, the wells were blocked by 300 µl of 2% of BSA in PBS at 4°C overnight. The plate was again washed twice. For detection of HEV in monkey stool, 100 µl of a 20% (w/v) stool suspension of the experimentally
- 30 infected monkey was added to a well. After incubation at 37°C for 1 hour and washing 5 times, 100 µl of HRP-conjugated monkey anti-E2 (1:4000) was added, and the well was further incubated at 37°C for 30 minutes and washed. 100 µl of TMB was added to the well and incubated at 37°C for 15 minutes. The colour development was stopped by the addition of 100 µl of 0.3 M H₂SO₄. The plate was read at an

absorbance of 450 nm.

Example 8

The Role of E2 in HEV Protection

5

A. Animals

Wild monkeys, rhesus macaques, were quarantined for 1 month and then bled for a test of ALT and HEV antibodies. The monkeys with ALT/AST over 60 or IgG anti-HEV positive were excluded from the study. The monkeys recruited were divided into three groups, one test group and one control group. Each group consisted of three animals.

10

B. Immunization

The test group was immunized with 100 μ g of E2 in a one-week interval. The first dose contained an equal volume of complete freund adjuvant. Incomplete freund adjuvant was used in subsequent doses. When the specific antibodies were elicited to a satisfactory level, the animals were then given an I/V booster. The control group underwent the same process with the exception that E2 was replaced by a placebo.

20

C. Challenge of HEV Particles

On the 20th day after the booster, both the test and control groups of animals were challenged with 10 μ l of monkey bile which contained HEV particles.

25

D. Monitoring of Primates

Alanine aminotransferase (ALT) levels, HEV-specific antibodies, viremia, virus loading in PMBC and stool shedding were monitored as described below:

30

1. Virus Loading in Peripheral Mononuclear Blood Cell (PMBC): PMBC was collected once a week prior to challenge and three times a week for four

w eks thereafter. Viral RNA in PMBC was extracted with QIAmp RNA blood minikit [QIAGEN, Germany] according to manufacturer's instructions. The process for coll ction was as follows: (a) 6 to 8 ml blood was collected in a 15 ml tube containing 10 µl heparin sodium [Cellimited, Australia]; (b) the blood was transferred gently to a 15 ml tube containing 5 ml ficoll [Pharmacia, Sweden]; (c) the tube was centrifuged at 2500 rpm for 15 minutes; (d) after centrifugation, the PMBC was separated at the upper interface of ficoll and the plasma and PMBC were separately and carefully transferred to new tubes; and (e) PMBC was washed three times with Hank's Balanced Salt Solution (5.4 mM KCl, 0.3 mM Na₂HPO₄, 0.4 mM KH₂PO₄, 4.2 mM NaCO₃, 1.3 mM CaCl₂, 0.5 mM MgCl₂, 0.6 mM MgSO₄, 137 mM NaCl, 5.6 mM D-glucose, 0.02% phenol red, pH 7.4) and stored with 20% FSC, 10% DMSO in 1640 RPMI medium at -70°C.

2. Level of Alanine Aminotransferase (ALT): Fresh sera or plasma was assayed by a 7170 Automatic Analyzer [HITACHI, Japan] for levels of ALT.

3. Antibody Response: HEV-specific antibodies were detected with two commercial HEV ELISA kits [Genelabs Diagnostics, Singapore and Beijing Medical University, China] and a ELISA test based on E2.

4. Stool Shedding: Stool samples were collected once before a challenge and daily thereafter. Virus shedding was detected using IC-RT-PCR and sandwich ELISA as described above.

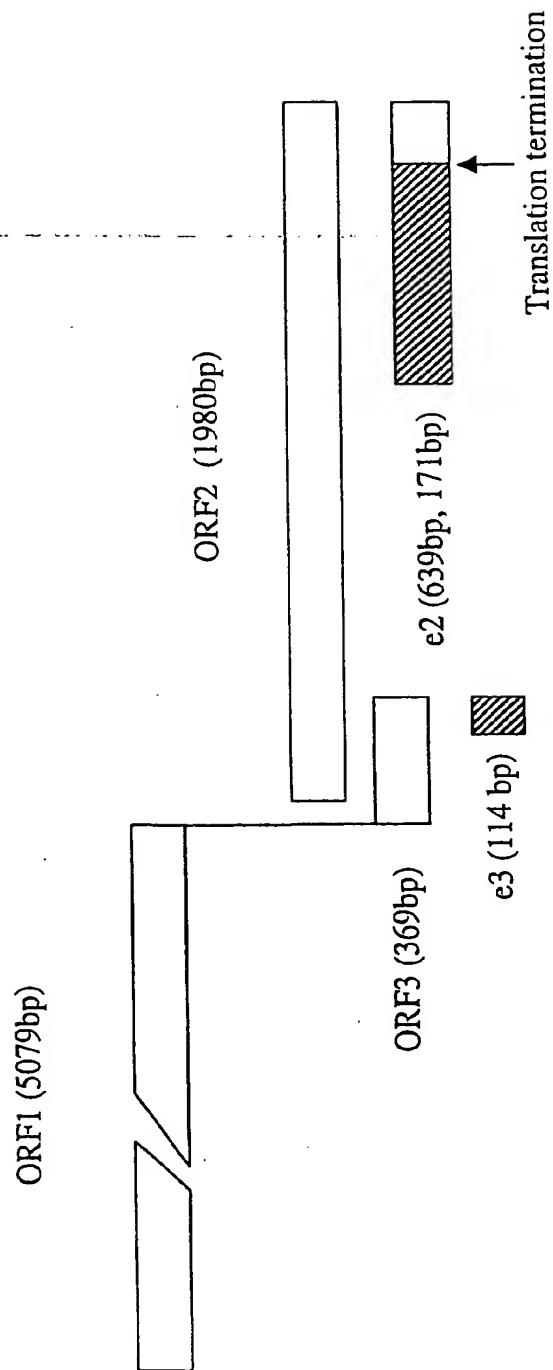


Figure 1

ORF2 5147 ATGCGCCCTCGGCCTATTTTGCTGTTGCTCCTCATGTTTCTGCCTATGCT
 v10 v v v v
 ORF2 5197 GCCCGCGCCACCGCCCGGTCAGCCGTCTGGCCGCGTCGTGGGCGGCGCA
 v60 v v v v
 ORF2-5247-GEGGEGGTTCGGGEGGTGGTTTCTGGGGTGACCGGGTTGATTCTCAGCCC
 v110 v v v v
 ORF2 5297 TTCGCAATCCCCTATATTCATCCAACCAACCCCTTCGCCCCCGATGTCAC
 v160 v v v v
 ORF2 5347 CGCTGCGGCCGGGGCTGGACCTCGTGTTTCGCCAACCCGCCCCGACCACTCG
 v210 v v v v
 ORF2 5397 GCTCCGCTTGGCGTGACCAGGCCAGCGCCCCGCCGTTGCCTCACGTGCT
 v260 v v v v
 ORF2 5447 AGACCTACCACAGCTGGGGCCGCGCCGCTAACCGCGGTCGCTCCGGCCCA
 v310 v v v v
 ORF2 5497 TGACACCCCGCCAGTGCCTGATGTTGACTCCCGCGGCGCCATCCTGCGCC
 v360 v v v v
 ORF2 5547 GGCAGTATAACCTATCAACATCTCCCTTACTTCTTCCGTGGCCACCGGT
 v410 v v v v
 ORF2 5597 ACAAACTTGTTTCTATACGCCGCTCCTCTTAGCCCACTTCTACCCCTCCA
 v460 v v v v
 ORF2 5647 GGACGGCACCAATACTCATATAATGGCCACAGAAGCTTCTAATTATGCCC
 v510 v v v v
 ORF2 5697 AGTACCGGGTTGCTCGTGCCACAATTCGCTACCGCCCGCTGGTCCCCAAC
 v560 v v v v
 ORF2 5747 GCTGTTGGTGGCTACGCCATCTCCATCTCGTTCTGGCCACAGACCACCAC
 v610 v v v v
 ORF2 5797 CACCCCGACGTCCGTTGACATGAATTCAATAACCTCGACGGATGTTTCGTA
 v660 v v v v
 ORF2 5847 TTTTAGTCCAGCCCGGCATAGCCTCCGAGCTTGTTATCCCAAGTGAGCGC
 v710 v v v v

Figure 2A

```

      v760      v      v      v      v
ORF2 5897 CTACACTACCGTAACCAAGGTTGGCGCTCTGTTGAGACCTCCGGGGTGGC

      v810      v      v      v      v
ORF2 5947 GGAGGAGGAGGCCACCTCTGGTCTTGTTATGCTCTGCATACATGGCTCAC

      v860      v      v      v      v
ORF2 5997 CTGTAAATTCTTATACTAATACACCTTATACCGGTGCCCTCGGGCTGTTG

      v910      v      v      v      v
ORF2 6047 GACTTTGCCCTCGAACTTGAGTTCCGCAACCTCACCCCGGTAATACCAA

      v960      v      v      v      v
ORF2 6097 CACGCGGGTCTCCCGTTACTCCAGCACTGCCCCGTCACCGCCTTCGTCGCG

      v1010     v      v      v      v
ORF2 6147 GTGCAGATGGGACTGCCGAGCTTACCACCACGGCTGCTACCCGCTTCATG

      v1060     v      v      v      v
ORF2 6197 AAGGACCTCTATTTTACTAGTACTAATGGTGTGCGGTGAGATCGGCCGTGG

      v1110     v      v      v      v
ORF2 6247 GATAGCGCTTACCCTGTTTAACTTGCTGACACCCTGCTTGGCGGTCTAC

      v1160     v      v      v      v
ORF2 6297 CGACAGAATTGATTTTCGTGCGGTGGTGGCCAGCTGTTCTACTCTCGTCCC
e2      1      CAGCTGTTCTACTCTCGTCCC

      v1210     v      v      v      v
ORF2 6347 GTCGTCTCAGCCAATGGCGAGCCGACTGTTAAGCTTTATACATCTGTAGA
e2      22      GTCGTCTCAGCCAATGGCGAGCCGACTGTTAAGCTTTATACATCTGTAGA

      v1260     v      v      v      v
ORF2 6397 GAATGCTCAGCAGGATAAGGGTATTGCAATCCCGCATGACATCGACCTCG
e2      72      GAATGCTCAGCAGGATAAGGGTATTGCAATCCCGCATGACATCGACCTCG

      v1310     v      v      v      v
ORF2 6447 GGGAGTCTCGTGTAGTTATTTCAGGATTATGACAACCAACATGAGCAGGAC
e2      122     GGGAGTCTCGTGTAGTTATTTCAGGATTATGACAACCAACATGAGCAGGAC

```

Figure 2B

```

                v1360      v      v      v      v
ORF2 6497 CGACCGACACCTTCCCCAGCCCCATCGCGCCCTTTTTCTGTCTCCTCCGAGC
          .....
e2    172 CGACCGACACCTTCCCCAGCCCCATCGCGCCCTTTTTCTGTCTCCTCCGAGC

                v1410      v      v      v      v
ORF2 6547 TAATGATGTGCTTTGGCTTTCTCTCACCGCTGCCGAGTATGACCAGTCCA
          .....
e2    222 TAATGATGTGCTTTGGCTTTCTCTCACCGCTGCCGAGTATGACCAGTCCA
          .....

                v1460      v      v      v      v
ORF2 6597 CTTACGGCTCTTCGACCGGCCAGTCTATGTCTCTGACTCTGTGACCTTG
          .....
e2    272 CTTACGGCTCTTCGACCGGCCAGTCTATGTCTCTGACTCTGTGACCTTG

                v1510      v      v      v      v
ORF2 6647 GTTAATGTTGCGACCGGCGCGCAGGCCGTTGCCCGGTCACTCGACTGGAC
          .....
e2    322 GTTAATGTTGCGACCGGCGCGCAGGCCGTTGCCCGGTCACTCGACTGGAC

                v1560      v      v      v      v
ORF2 6697 CAAGGTCACACTTGATGGTCGCCCCCTTTCCACCATCCAGCAGTATTCAA
          .....
e2    372 CAAGGTCACACTTGATGGTCGCCCCCTTTCCACCATCCAGCAGTATTCAA

                v1610      v      v      v      v
ORF2 6747 AGACCTTCTTTGTCCTGCCGCTCCGCGGTAAGCTCTCCTTTTGGGAGGCA
          .....
e2    422 AGACCTTCTTTGTCCTGCCGCTCCGCGGTAAGCTCTCCTTTTGGGAGGCA

                v1660      v      v      v      v
ORF2 6797 GGTACTACTAAAGCCGGGTACCCTTATAATTATAACACCACTGCTAGTGA
          .....
e2    472 GGTACTACTAAAGCCGGGTACCCTTATAATTATAACACCACTGCTAGTGA

                v1710      v      v      v      v
ORF2 6847 CCAACTGCTCGTTGAGAATGCCGCTGGGCATCGGGTTGCTATTTCCACTT
          .....
e2    522 CCAACTGCTCGTTGAGAATGCCGCTGGGCATCGGGTTGCTATTTCCACTT

                v1760      v      v      v      v
ORF2 6897 ACACCACTAGCCTGGGTGCTGGTCCCGTCTCTATTTCCGCGGTTGCTGTT
          .....
e2    572 ACACCACTAGCCTGGGTGCTGGTCCCGTCTCTATTTCCGCGGTTGCTGTT

```

Figure 2C

ORF2 6947 TTAGCCCCCCTCTCCGCGCTAGCATTGCTTGAGGATACCATGGACTACCC
e2 622 TTAGCCCCCCTCTCCGCGCTAGCATTGCTTGAGGATACCATGGACTACCC
Stop code

ORF2 6997 TGCCCGCGCCCATACTTTCGATGACTTCTGCCCCGAGTGCCGCCCCCTTG
e2 672 TGCCCGCGCCCATACTTTCGATGACTTCTGCCCCGAGTGCCGCCCCCTTG

ORF2 7047 GCCTCCAGGGCTGTGCTTTTCAGTCTACTGTCGCTGAGCTTCAGCGCCTT
e2 722 GCCTCCAGGGCTGTGCTTTTCAGTCTACTGTCGCTGAGCTTCAGCGCCTT

ORF2 7097 AAGATGAAGGTGGGTAAAACTCGGGAGTTATAGTTTATTGCTTGCGCC
e2 772 AAGATGAAGGTGGGTAAAACTCGGGAGTTATAGTTTATT

ORF2 7147 CCCTTCTTTCTGTTGCTTATTTCTTTTCTGCGTTCCGCGCTCCCTGAAA

ORF2 7197 AAA

Figure 2D

```

      v10      v      v      v      v
ORF3 5106 ATGAATAACATGTCTTTTGCTGCGCCCATGGGTTCGCGACCATGCGCCCT

      v60      v      v      v      v
ORF3 5156 CGGCCTATTTTGCTGTTGCTCCTCATGTTTCTGCCTATGCTGCCCGCGCC

      v110     v      v      v      v
ORF3 5206 ACCGCCCCGGTCAGCCGTCTGGCCGCCGTCTGTGGGCGGCGCAGCGGCGGTT

      v160     v      v      v      v
ORF3 5256 CCGGCGGTGGTTTCTGGGGTGACCGGGTTGATTCTCAGCCCTTCGCAATC

      v210     v      v      v      v
ORF3 5306 CCCTATATTCATCCAACCAACCCCTTCGCCCCCGATGTCACCGCTGCGGC

      v260     v      v      v      v
ORF3 5356 CGGGGCTGGACCTCGTGTTTCGCCAACCCGCCCCGACCACTCGGCTCCGCTT
      ::::::::::::::::::::::::::::::::::::::::::::::::::::::
e3      1      GACCTCGTGTTTCGCCAACCCGCCCCGACCACTCGGCTCCGCTT

      v310     v      v      v      v
ORF3 5406 GGCGTGACCAGGCCAGCGCCCCGCCGTTGCCTCACGTCGTAGACCTACC
      ::::::::::::::::::::::::::::::::::::::::::::::::::::::
e3      51     GGCGTGACCAGGCCAGCGCCCCGCCGTTGCCTCACGTCGTAGACCTACC

      v360     v
ORF3 5456 ACAGCTGGGGCCGCGCCGCTAA
      ::::::::::::::
e3      101    ACAGCTGGGGCCGCGCCGCTAA

```

Figure 3

v10 v v v v
E2 1 QLFYSRPVVSANGEPTVKLYTSVENAQQDKGIAIPHDIDLGESRVVIQDY

 v60 v v v v
E2 51 DNQHEQDRPTPSPAPSRPFSVLRANDVLWLSLTAAEYDQSTYGSSTGPVY

 v110 v v v v
E3 101 VSDSVTLVNVATGAQAVARSLDWTQVTLTLDGRPLSTIQQYSKTFVFLPLRG

 v160 v v v v
E3 151 KLSFWEAGTTKAGYPYNYNTTASDQLLVENAAGHRVAISTYTTSLGAGPV

 v210
E3 201 SISAVAVLAPPPR

Figure 4



E3 1 DLVFANFPDHSAPLGVTRPSAPPLPHVVDLPQLGPRR.

Figur 5

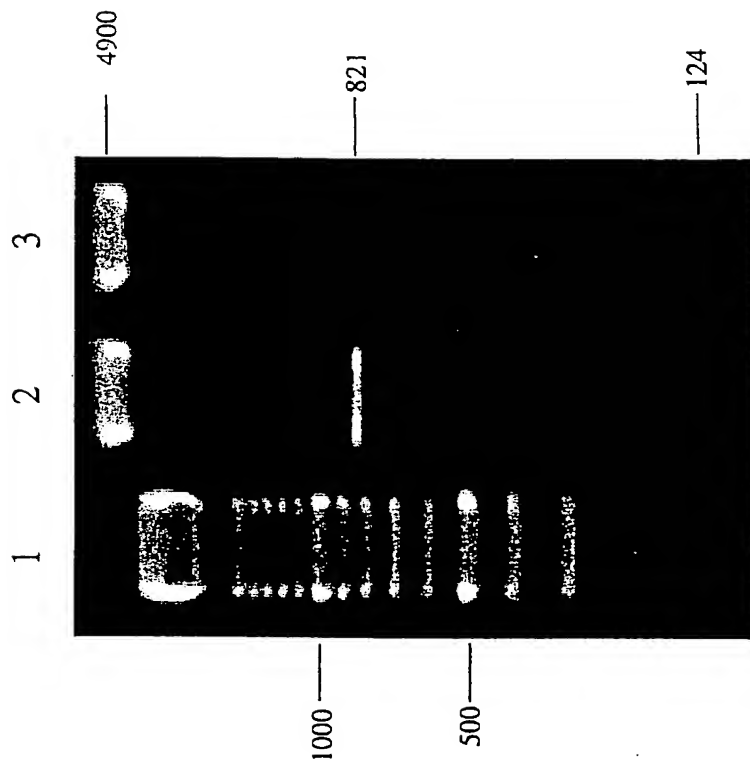


Figure 6

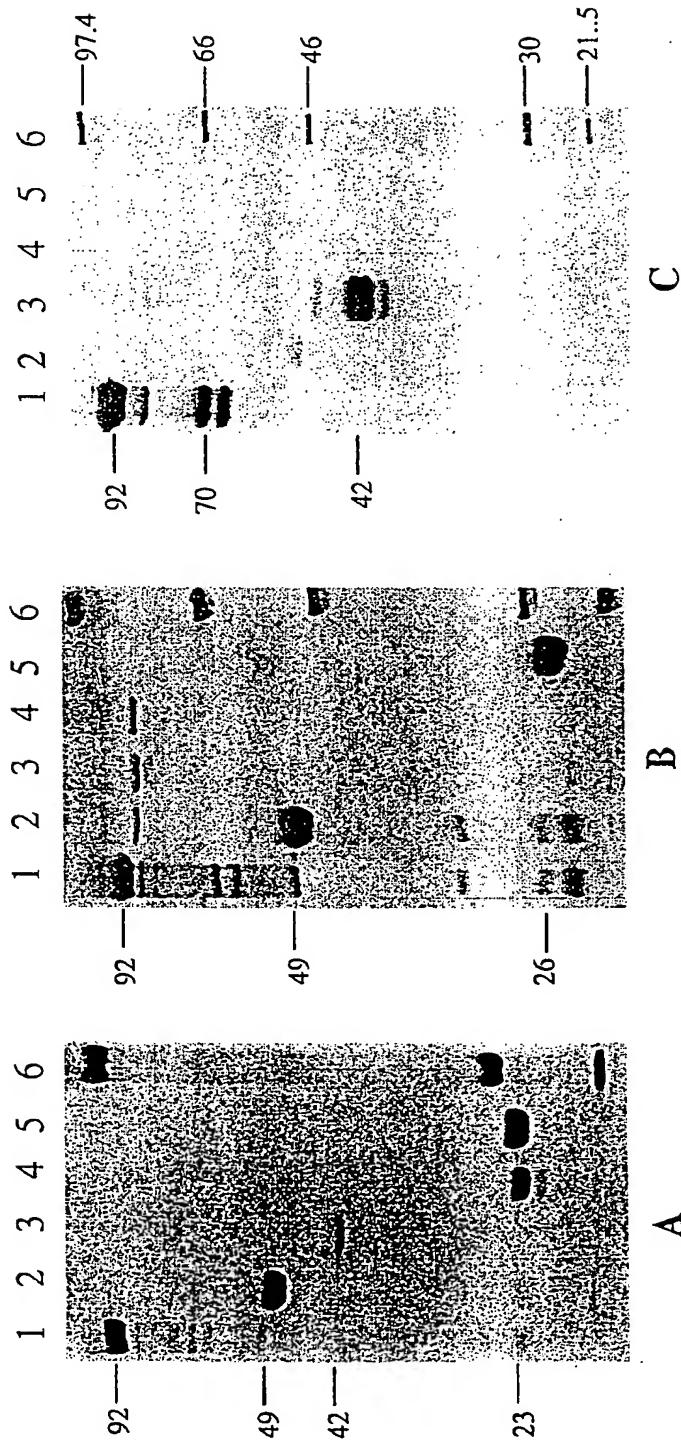


Figure 7

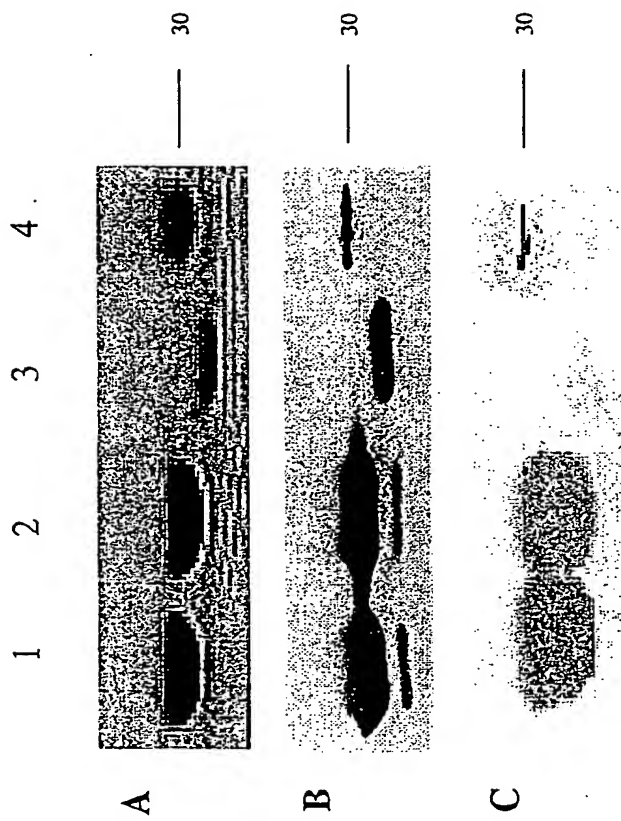


Figure 8

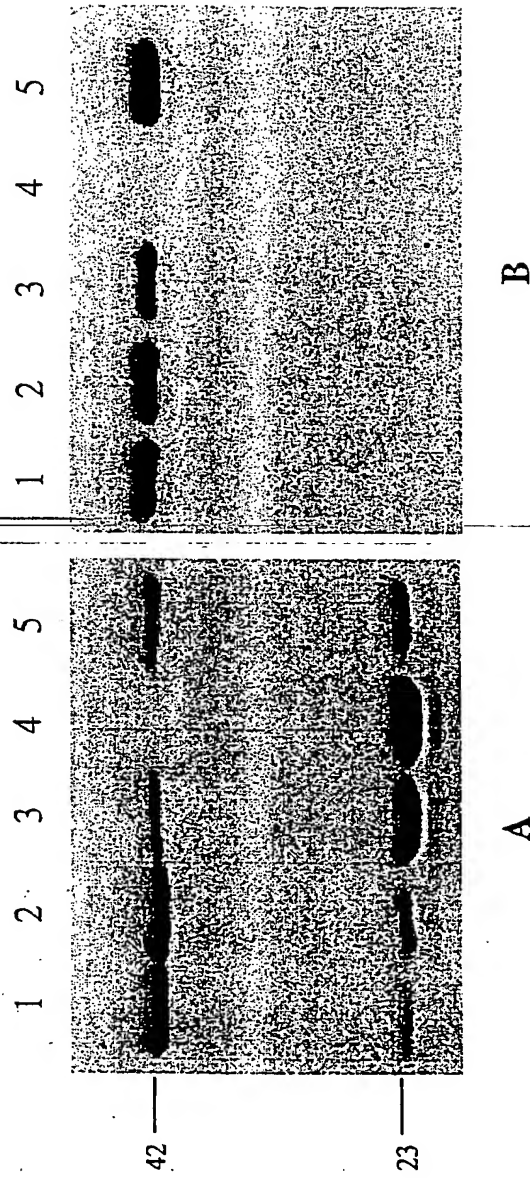
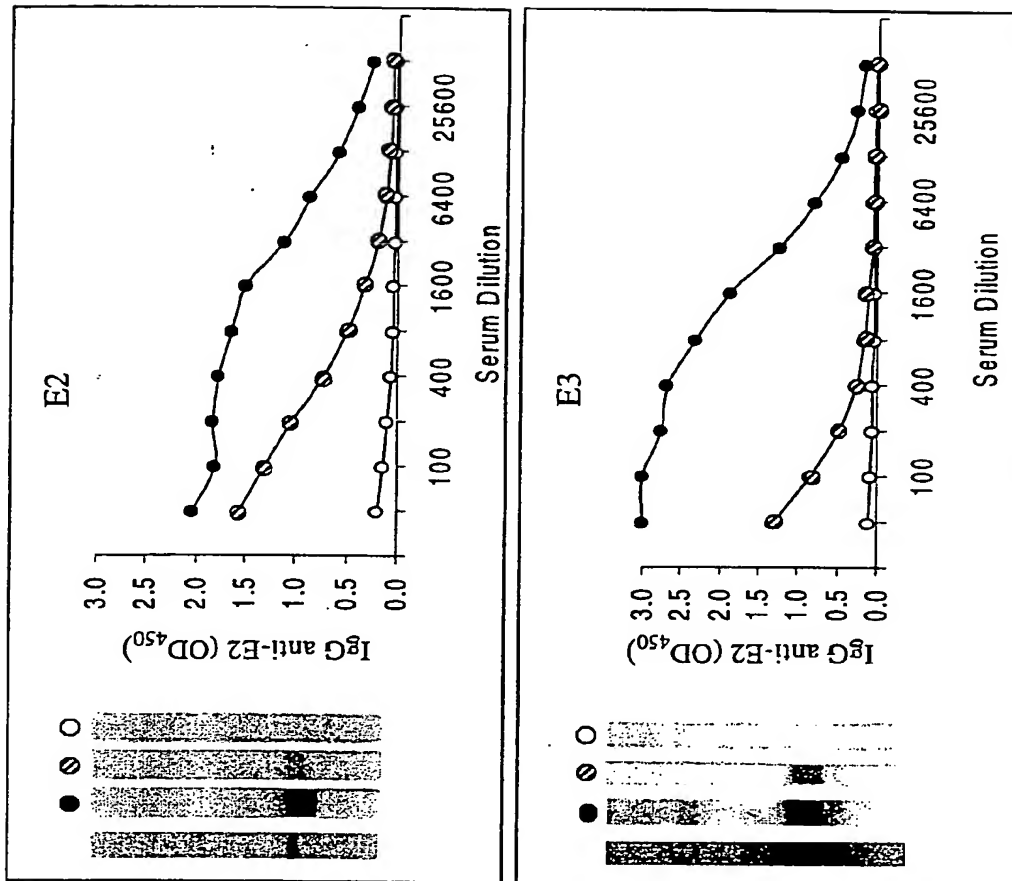


Figure 9

**Figure 10**

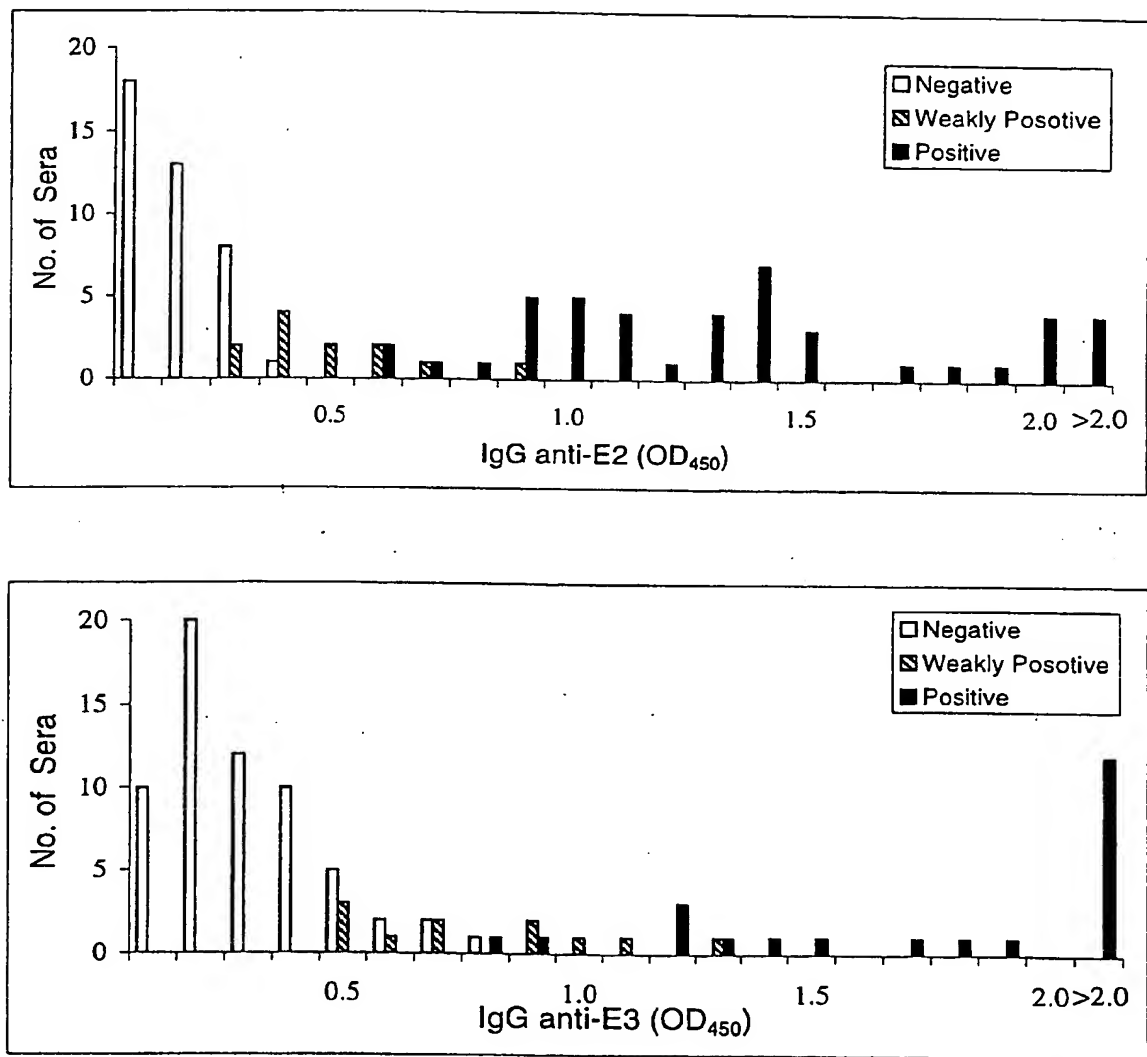
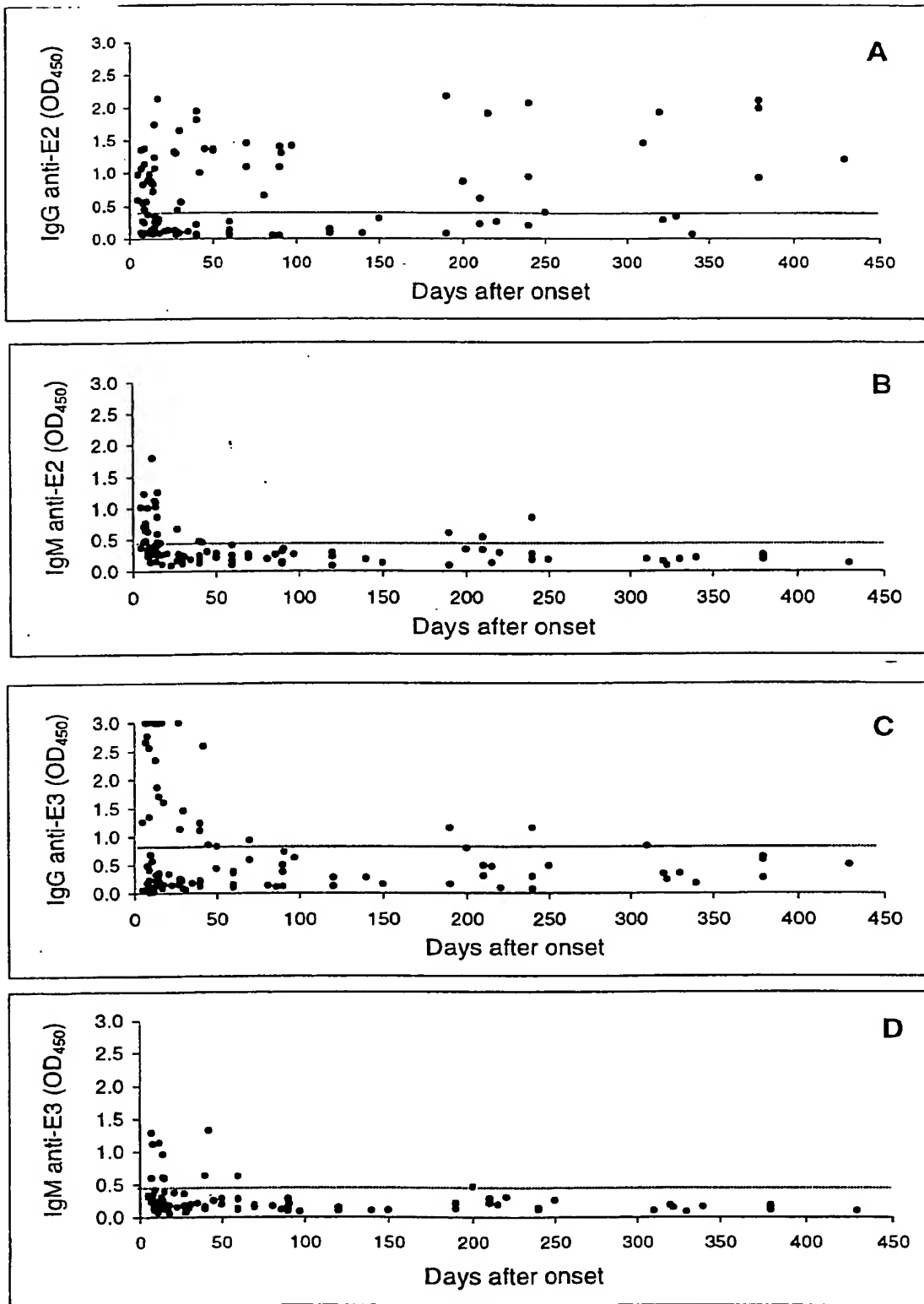
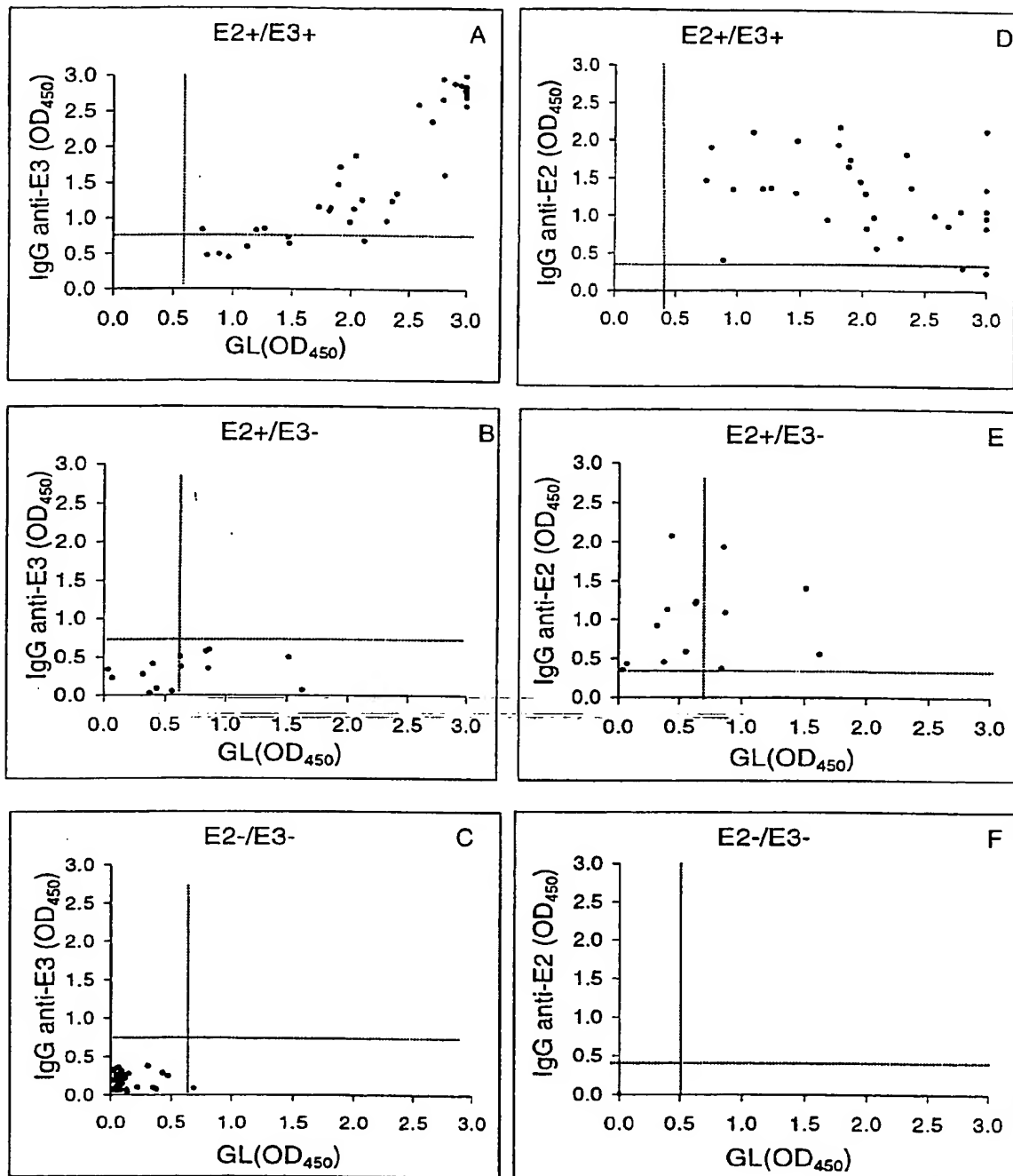
**Figure 11**

Figure 12



**Figure 13**

1 2 3 4 5 6 7



Figure 14

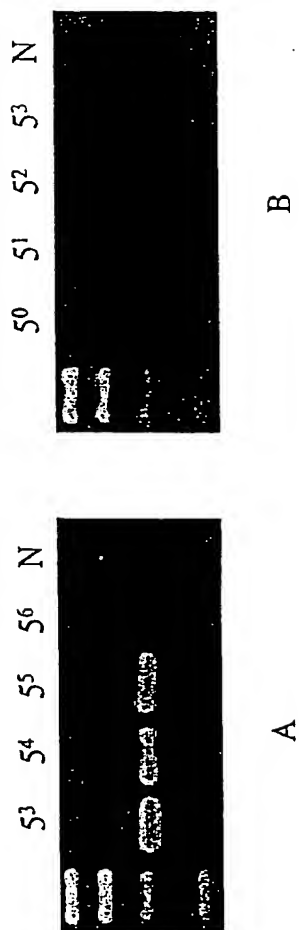


Figure 15

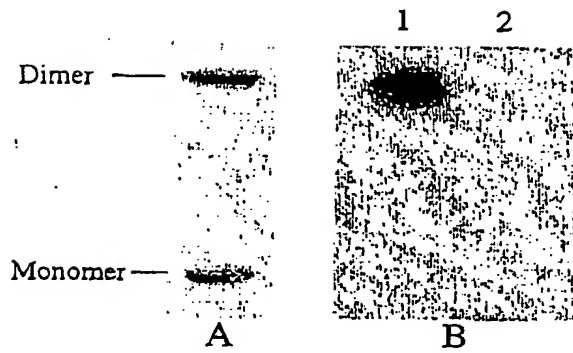
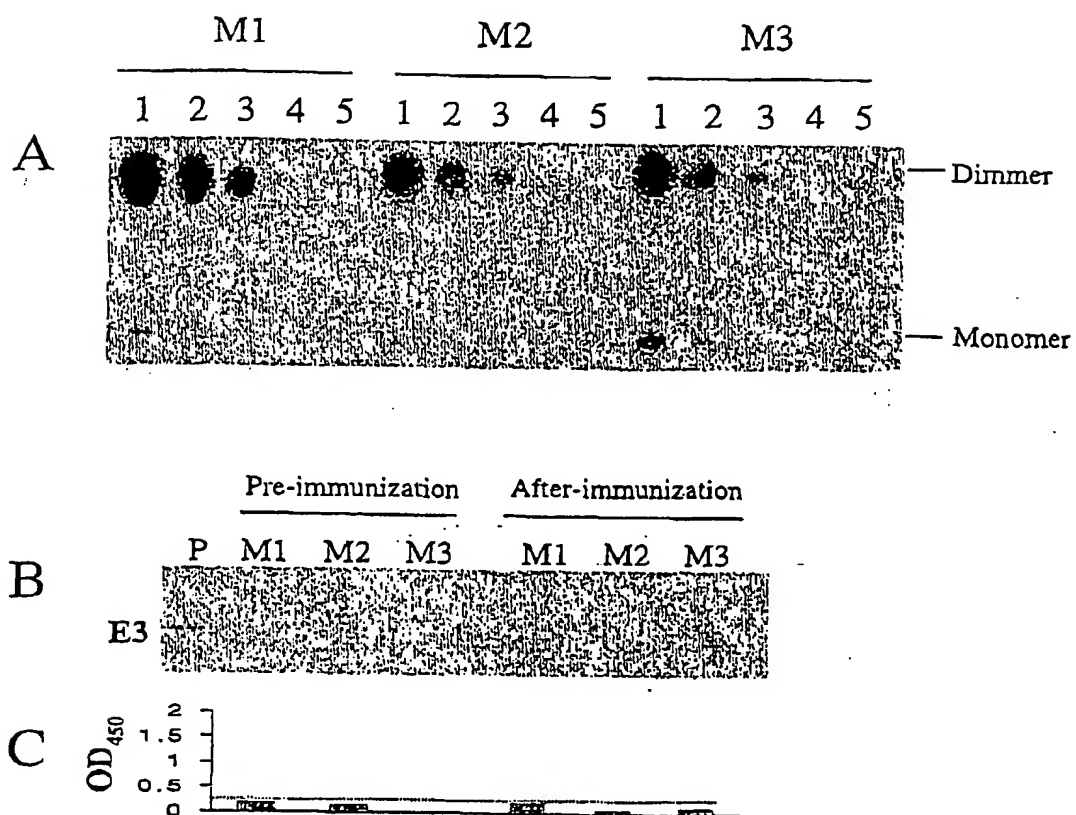


Figure 16

**Figure 17**

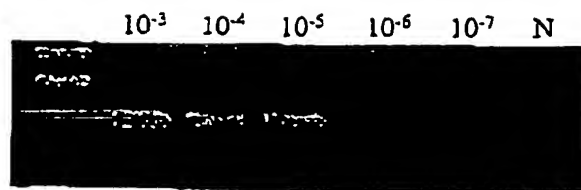
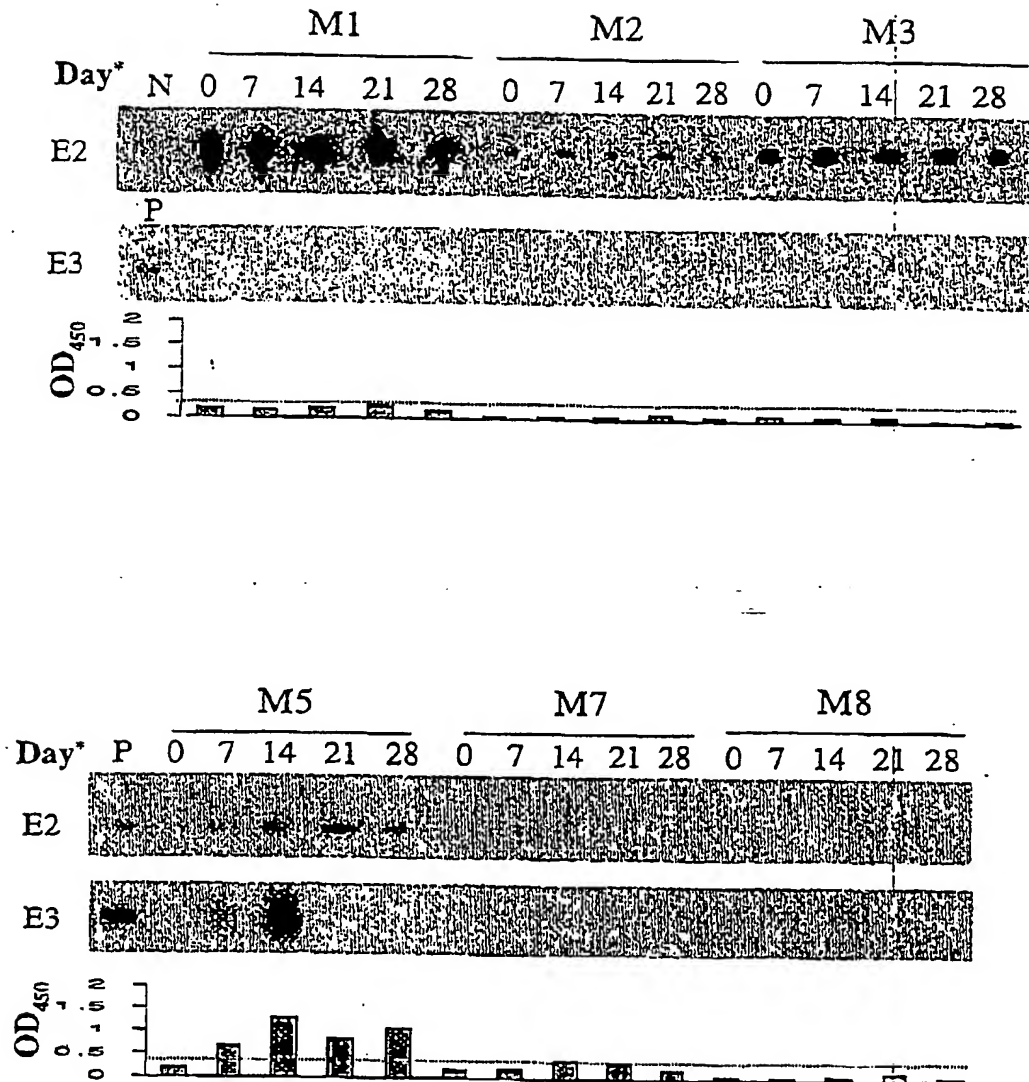
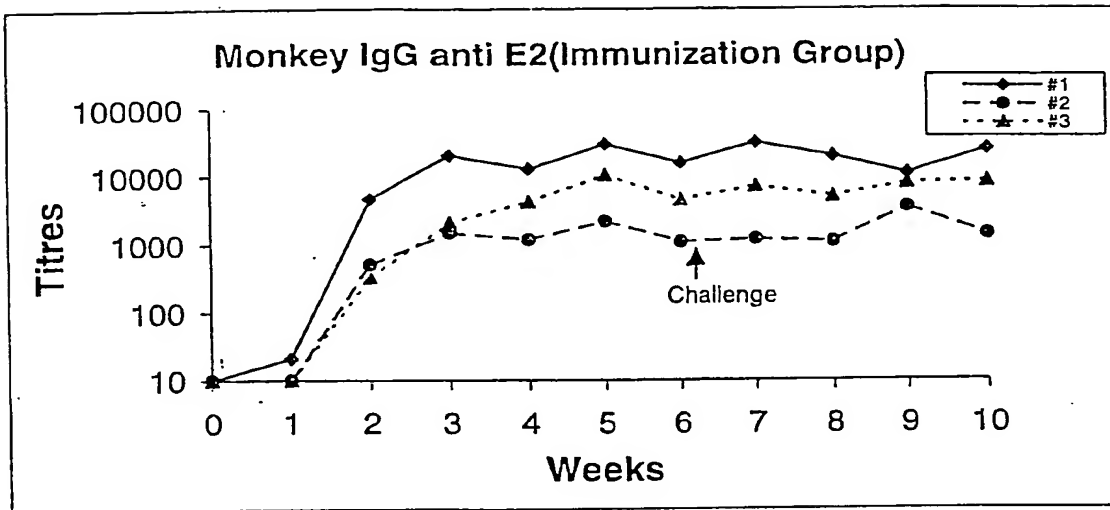
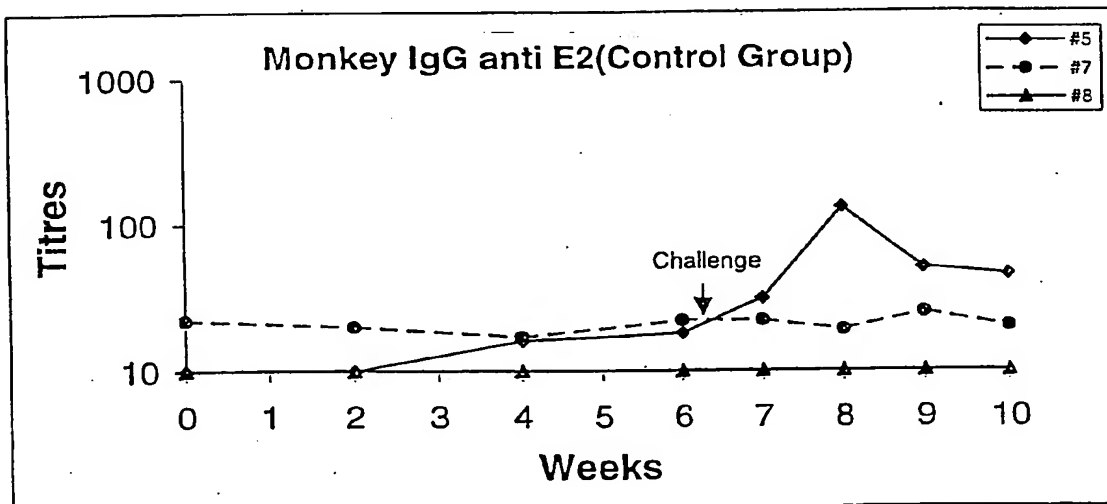


Figure 18

**Figure 19**

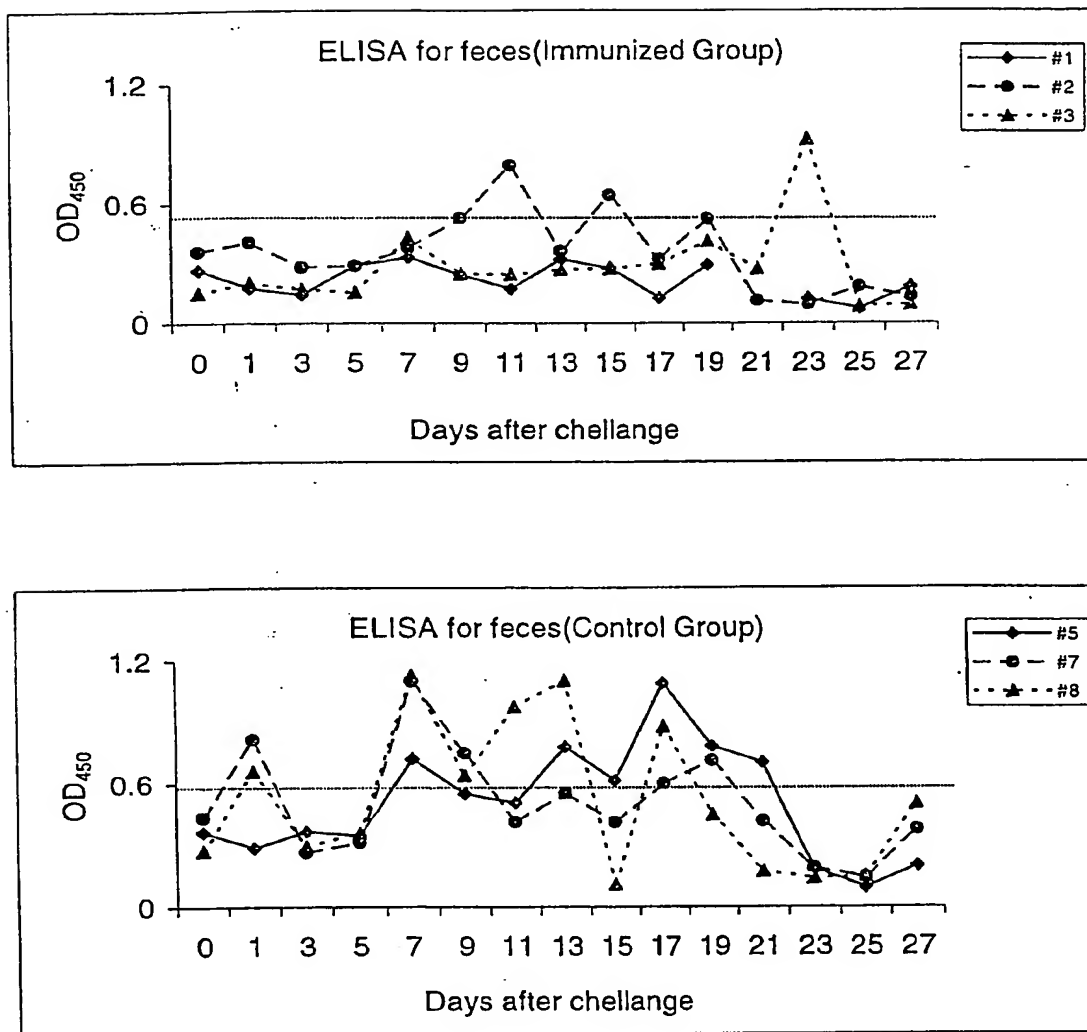


A



B

Figure 20

**Figure 21**